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DEVELOPMENT OF OIL IN THE SEED OF HELIANTHUS ANNUUS L.[1]

C. Y. HOPKINS AND MARY J. CHISHOLM

Abstract

Sunflower seed (*Helianthus annuus* L.) was collected from growing plants at weekly intervals. Analyses of the seed, the oil, and the fatty acids were made, the latter by gas chromatography. Oil formation began about 10 days after flowering and continued at a steady rate for 7 weeks. There was no evidence that any intermediate substance accumulated in the seed for subsequent conversion to oil.

The weight of individual fatty acids per 100 seeds was determined at each stage of development. Except for one sample, the amount of each acid increased to maturity. Oleic acid accumulated most rapidly at first but at the mid-point of oil development it was overtaken by linoleic acid, which became the major component. The possibility that some oleic acid was converted to linoleic acid is discussed. The absence of any marked change in amounts of long-chain saturated acids suggests that they were not involved in the synthesis of the unsaturated fatty acids.

Introduction

The development of fatty oil in seeds during maturation has been studied in various species with the object of determining the route of biosynthesis of the fatty acids. Only a few of these studies have included a quantitative analysis of the fatty acids, without which the results are of limited value. The introduction of gas chromatography has made it possible to perform such analyses conveniently and with small samples. Accordingly, the present investigation was undertaken to examine the formation of oil in the sunflower seed.

The properties of oil from sunflower seed harvested at intervals during maturation were reported by Bauer in 1934 (1). He carried out also a partial analysis of the fatty acids by the thiocyanate method. Vidal made an examination of oil from sunflower seeds collected over a growing period of 1 month (2). A more extensive study was made by Jáky and Homonnayné (3) in 1957, but

[1]Manuscript received February 20, 1961.
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neither they nor Vidal determined the individual fatty acids. Dubinskaya collected sunflower seed at 5-day intervals and analyzed the fatty acids by the method of ultraviolet absorption after alkali isomerization (4). In a similar study, Franzke (5) determined the saturated acids by a lead salt separation and linoleic acid by ultraviolet absorption analysis. Oleic acid was estimated by difference and the amounts of each acid per gram of seed were calculated.

The present work includes the determination of amounts of each component fatty acid per 100 seeds, in order to follow the accumulation of individual acids during the period of oil formation.

Methods and Results

Sunflower seed (*Helianthus annuus* L., var. Advance) was sown at Ottawa on May 21, 1959. This is a semidwarf, early maturing variety developed in Western Canada. Flowering was general on July 28. As soon as pollination was complete, the flower heads were covered with perforated paper bags to protect the seed from birds. Growth and yield were satisfactory but there was some formation of mold or fungus on the seeds at the end of the season during wet weather.

Two heads were harvested approximately every seven days, beginning on August 7. The seeds were not allowed to ripen but were removed from the heads, pooled, and dried at 110° on the day of harvesting.

Analysis of Seed and Oil

The whole seeds were ground in a Wiley mill and the oil was extracted quantitatively in a Soxhlet apparatus with petroleum ether (b.p. 30–60°). The moisture content and oil content of the seeds are shown in Table I.

TABLE I

Composition of seed and oil

Date of harvest	Moisture (wt. %)	Oil content, dry basis (wt. %)	Free acid as oleic (wt. % of the oil)	Unsaponifiable matter (wt. % of the oil)
Aug. 7	89.8	6.5	—	—
Aug. 14	73.9	10.6	2.2	4.3
Aug. 21	71.4	19.6	0.9	1.8
Aug. 28	68.3	16.7	1.1	1.7
Sept. 4	55.1	19.5	1.1	1.4
Sept. 14	26.8	22.3	1.3	1.2
Sept. 18	32.0	21.1	1.0	1.2
Sept. 25	20.1	25.8	2.6	1.4
Oct. 2	24.9	27.1	4.5	1.5

The seeds gained weight during the first 2 weeks and then lost weight as the moisture content decreased rapidly while the non-volatile matter (including oil) increased more slowly. There was some fluctuation of moisture content during the last month because of alternating dry and rainy weather. The weight of oil per 100 seeds is shown in Fig. 1.

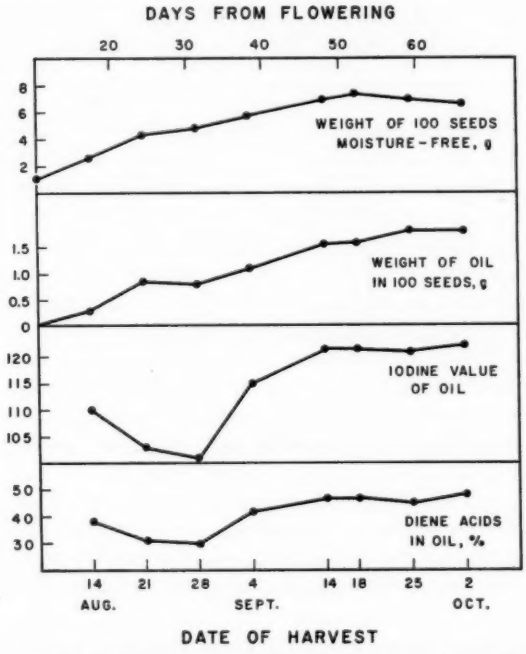

FIG. 1. Amount and unsaturation of the oil at various stages of maturity.

The oil was analyzed by official A.O.A.C. methods (6). The August 14 sample was somewhat higher than usual in free acid and unsaponifiable matter, but succeeding samples were normal in these properties up to the last 2 weeks. The increase in acid value at that time may have been due to fungus or mold growth on the seeds.

The most noticeable change was the sudden increase in diene content and iodine value of the oil between August 28 and September 4 (Fig. 1). The mean equivalent weight of the fatty acids showed little change over the period studied.

Composition of the Fatty Acids

The oils were saponified and the unsaponifiable matter was removed. The mixed fatty acids were converted to methyl esters and analyzed by gas chromatography, using a diethylene glycol – succinic acid polyester as the liquid phase. Column length was 1 meter, temperature 190°, and helium flow rate 95 ml/minute, at an inlet pressure of 13 p.s.i. The detector was a thermistor type of thermal conductivity cell and the recorder span was 0–5 millivolts. Four chromatograms were made from each lot of esters and the percentage composition of the fatty acids was determined by measuring the peak areas. The means of these results are shown in Table II.

TABLE II

Percentage composition of the fatty acids (by gas chromatography)*

Date of harvest	Palmitic†	Stearic	Oleic	Linoleic
Aug. 14	10.0	2.8	41.5	45.7
Aug. 21	5.8	1.5	55.0	37.7
Aug. 28	4.2	1.3	57.4	37.1
Sept. 4	4.4	1.6	45.3	48.7
Sept. 14	4.3	1.6	38.8	55.3
Sept. 18	4.3	1.5	41.8	52.4
Sept. 25	4.3	1.2	41.3	53.2
Oct. 2	4.8	1.0	39.8	54.4

*Probable limit of error ± 0.5 units %.
†Includes a small proportion of hexadecenoic acid.

The peaks were identified by their emergence times, in comparison with standard samples, as those of palmitic, stearic, oleic, and linoleic esters. The chromatograms indicated the presence of a trace of hexadecenoic acid but there was no evidence of linolenic acid or of C_{20} acids.

Two samples of the oil were examined for minor constituents. Ultraviolet absorption analysis showed that the content of conjugated acids was negligible (0.2%). The content of oxirane oxygen was also negligible (0.01%), showing that there was no measurable amount of epoxy acid. Hydroxyl content was less than 0.1%.

Marked changes in composition took place, as shown by the data in Table II. The percentage of saturated acids decreased while that of oleic fluctuated considerably. The percentage of linoleic acid decreased after the first sampling and then increased to over 50% of the total acids. There was little change in composition during the last 4 weeks.

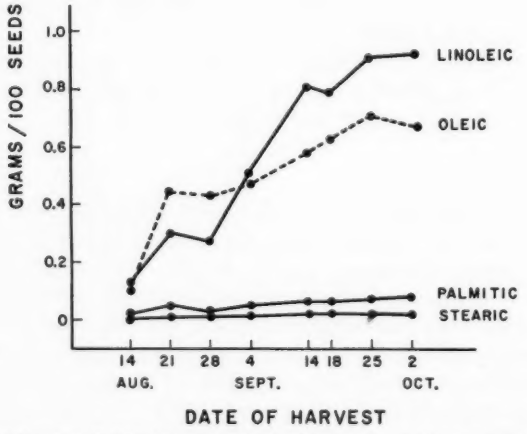

FIG. 2. Weight of individual fatty acids in 100 seeds at various stages of maturity.

The amounts of accumulated individual acids per 100 seeds were calculated and are shown graphically in Fig. 2.

Discussion and Conclusions

As shown in Fig. 1, the weight of oil per 100 seeds continued to increase over a period of 7 weeks. Other species of annual plants that have been studied have a shorter period of oil deposition. In flax (7), oil deposition began about the 8th day after flowering and was practically complete in 3 weeks. Similarly, in cotton (8) and soybean (9), although oil formation began much later it was substantially complete in 3 weeks.

The weight of non-volatile matter, other than oil, in the seed can be calculated from Table I. It increased steadily along with the oil. Hence, it is unlikely that any intermediate substance, such as carbohydrate, could have accumulated in the seed during the early stages of maturation and been converted later to oil. If the oil is synthesized from carbohydrate in the seed, formation of carbohydrate and its conversion to oil must take place concurrently.

Characteristics of the Oil

The nature of the oil changed considerably during the growth of the seed. Most of the change occurred during the first month of oil formation (Table I, Fig. 1). The first sample to be analyzed was relatively high in acid value, unsaponifiable matter, and diene acid. These properties are typical of the small amount of immature seed lipid, referred to by Crombie as "basal protoplasmic fat" (10). One week later, the acid value and unsaponifiable matter had fallen to normal levels.

Between August 28 and September 4 a sudden increase in the iodine value took place, followed by a further increase in the following week (Fig. 1). A similar sharp rise in iodine value was noted in flax by Painter (7) and in cotton by Grindley (8), both at an early stage of oil development. As in the cottonseed, the increased iodine value in sunflower is evidently due to an increased proportion of diene acid (Fig. 1). The rise in iodine value was observed by nearly all of the earlier workers with sunflower (2, 3, 4, 5).

Composition of the Fatty Acids

The fatty acids of mature sunflower seed oil were shown by the analyses of Barker, Crossley, and Hilditch (11) to be mainly palmitic, stearic, oleic, and linoleic. These authors found also small amounts of hexadecenoic and arachidic acids but no linolenic acid. Our analysis by gas chromatography confirms these findings except that arachidic acid did not appear in our samples. Linolenic acid was absent and hexadecenoic acid was less than 1% of the total. Other tests showed the absence of conjugated and oxygenated acids. Accordingly, only the four main acids are shown in Table II.

Accumulation of Individual Acids

The amounts of each acid in grams per 100 seeds increased fairly regularly

to maturity on September 25. The sample collected on August 21 was an exception and there is no obvious explanation for its divergence from the trend. Figure 2 shows the rapid accumulation of oleic acid at an early stage and the change in rates of synthesis about September 1, when the rate of formation of linoleic acid increased sharply and exceeded that of oleic acid. Franzke (5) observed a similar inversion of rates of formation of oleic and linoleic acids in his study of sunflower, although his results were calculated as weight of acid per gram of seed.

It is evident that this is the stage of development at which formation of the characteristic seed oil of the species actually begins. In the palm kernel, Crombie (10) observed a corresponding stage at which the predominant oleic acid gave way to lauric acid in speed of formation.

The increase in amount of linoleic acid from August 28 to September 14 (Fig. 2) was quite large. The other acids show some increase over the same period but to a much lesser extent. There is no direct evidence of conversion of one acid to another during this period although the reduced rate of accumulation of oleic acid, concurrent with the rapid rise of linoleic acid, may indicate that some conversion of oleic to linoleic was taking place.

The pattern is much the same as in the conversion of labelled sucrose in the soybean, where the activity appeared first in the oleic acid and later in the linoleic acid, as reported by Simmons and Quackenbush (12). These observations provide support for the hypothesis that oleic acid is an intermediate in the formation of linoleic acid in the plant.

The reduction in amount of oleic acid which took place after September 25 was not accompanied by a significant increase in linoleic acid. It may be noted, however, that Franzke also observed a reduction in the weight of oleic acid (per gram of seed) near maturity (5).

The amounts of saturated long-chain acids remained small throughout and showed little change except for a fairly regular increase in the amount of palmitic. This behavior suggests that the saturated acids are not involved in the synthesis of the unsaturated acids.

Acknowledgments

The authors are indebted to Dr. F. Dimmock, Canada Department of Agriculture, for providing the plot and growing the plants; also to Dr. E. D. Putt for the supply of seed.

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MINOR PROTEINS IN HUMAN ERYTHROCYTES[1]

N. CHANDRASEKHAR[2]

(*Introduced by* L. B. JAQUES)

Abstract

A study of 200 normal human blood samples, by the agar electrophoresis method, revealed that human haemolyzates contain two minor proteins in addition to the normal adult haemoglobin. The two minor proteins appearing in very low concentrations are non-haemoglobin in character, give a negative reaction to benzidine test, but a positive reaction to amido-schwarz. These two proteins have an electrophoretic mobility similar to that of γ-globulin of human serum, but appear as sharp distinct bands on the agar plate. The concentration of these non-haemoglobin components bear no correlation to the blood groups of the samples or to the haemoglobin concentration itself, but the components have some electrophoretic characteristics similar to some of the abnormal haemoglobins.

Introduction

It has been customary to analyze the erythrocytes for the protein haemoglobin and its abnormalities. The clear red fluid obtained after haemolysis of the washed cells was taken as pure haemoglobin solution, and has been even employed for crystallization, alkali denaturation, and such other characterization purposes. Haurowitz *et al.* (1) reported the presence of two haemoglobins in rat blood, based on the discontinuities they observed in the alkali denaturation curves of rat blood haemolyzates. Giri and Pillai (2, 3) on agar electrophoretic analysis of rat blood have observed that rat blood cells contain two other minor protein components in addition to the haemoglobin. The same authors have made a similar observation (4) that human haemolyzates also contain another non-haemoglobin protein component. These findings prompted the author to investigate further about the occurrence of these minor non-haemoglobin proteins in normal human blood and their pattern in haemoglobin diseases. A study of 200 human blood samples carried out by the author is reported in this communication.

Experimental

Venous blood samples were obtained from normal humans and haemolyzates were prepared by the method of Drabkin (5) without using aluminum chloride. The cells were washed 6 times with sterilized normal saline to avoid any contamination of serum proteins in the haemolyzates. The blood haemolyzates were then analyzed by the agar electrophoresis technique (4). The haemoglobin bands could be observed visually and were confirmed by the benzidine test. The protein staining was carried out by amido-schwarz. The percentage

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composition of the haemoglobin and other protein components were evaluated by the use of a photovolt densitometer and by planimetry (6).

Results

Almost all the human blood samples on agar electrophoresis and subsequent staining with amido-schwarz revealed the presence of three protein components. The patterns are easily reproducible. Out of these only, one component gave a positive reaction to benzidine test, the normal adult haemoglobin A. The other two components, designated here as minor proteins (α, β), could not be detected by the benzidine test, though revealed by amido-schwarz. The two bands appear as clear, sharp lines and have an electrophoretic mobility similar to that of γ-globulin. A typical photograph of the agar electrophoretic pattern of normal human blood haemolyzate along with that of normal human serum is shown in Fig. 1.

In the 200 samples investigated, a very significant variation could be observed in the concentration of the minor proteins. Nearly 50% of the samples showed a measurable high intensity, whereas the rest of them varied from low to feebly noticeable concentrations. The maximum concentration observed in typical patterns of the human blood haemolyzates were haemoglobin, 96%; α, 2.5%; and β, 1.5%.

To see whether there is any correlation between concentration of these non-haemoglobin proteins and blood groups of the samples, a homogeneity χ^2 with 3 degrees of freedom was computed. The value obtained for χ^2 0.074 is highly insignificant, suggesting that there is no correlation between the two characters. The haemoglobin concentration also did not show any significant variation between the group with a low concentration of the minor proteins and the one with the high concentration of the minor proteins.

Discussion

Stern *et al.* (7) have, in a preliminary study of the blood haemolyzates of man, dog, and chicken, reported the presence of three new proteins occurring in very low concentrations. Hoch (8) has subsequently reported the occurrence of two components in erythrocyte extracts, with a mobility greater than that of oxyhaemoglobin. The two new proteins of human erythrocytes reported here are of considerably lower electrophoretic mobility than those reported by previous workers. In none of the samples investigated could any fast-moving component be observed. The fact that these two minor proteins are not any of the known serum proteins is shown by their remarkably sharp and well-defined appearance with a characteristically low mobility. It is interesting to note that the β-component has a mobility similar to that of haemoglobin E of the blood of thalassemic patients with the genetic haemoglobin E trait. The significant concentration variations of these two minor proteins in the different individuals is also noteworthy.

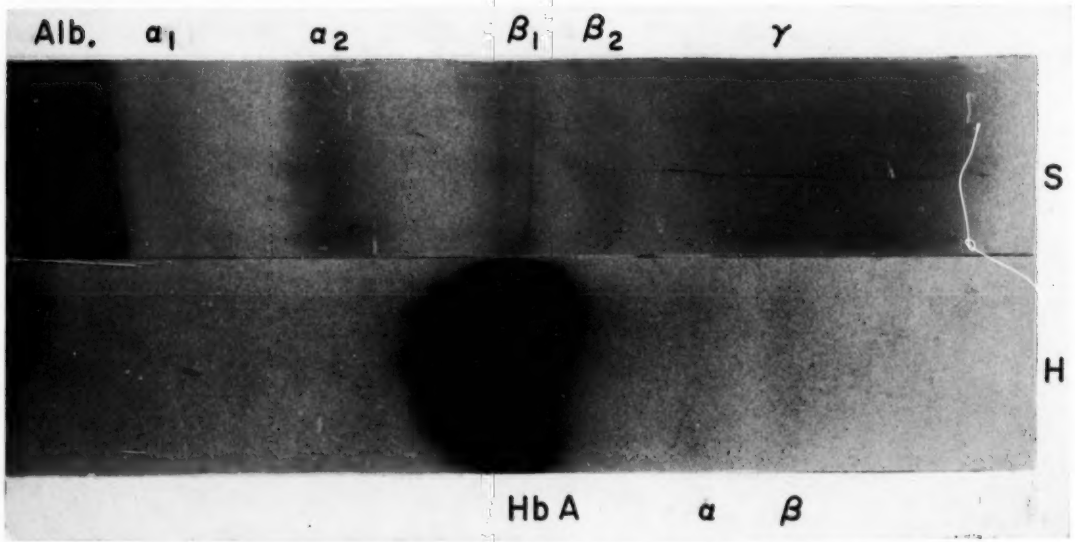

FIG. 1. Agar electrophoretic pattern of human serum along with human blood haemolyzate (200 v; 5 ma; 5-hour run; pH 8.6; I.S. 0.05). S = normal human serum; H = normal human haemolyzate.

It has been reported that the ABO blood groups bear a direct relationship to the occurrence of certain types of chronic diseases in humans (9, 10). Analysis of the data to find out whether there is any correlation between the blood groups and the intensity of concentration of the non-haemoglobin proteins revealed that the two characters are totally independent. The haemoglobin concentration was also found to bear no correlation with the concentration of the non-haemoglobin proteins. Hence it looks unlikely that these minor proteins may have any role in the production of normal haemoglobins; at the same time the possibility that these two new intracellular proteins may have some indirect role in the occurrence of abnormal haemoglobins or in the complex phenomenon of blood coagulation itself cannot be ruled out.

Acknowledgments

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THE REACTION OF MUSTARD GAS WITH DEOXYRIBONUCLEIC ACID WITH SPECIAL REFERENCE TO THE PYRIMIDINES[1]

I. G. WALKER AND W. J. WATSON

Abstract

Mustard gas was allowed to react with DNA at pH 7.4. After mild acid hydrolysis of the product, the mustard (estimated as sulphur) was found in three fractions: bound to purine, bound to apurinic acid, and a portion bound to neither and whose origin was not apparent. The apurinic acid was degraded to free pyrimidines by perchloric acid hydrolysis. From the hydrolyzate, a cytosine and a thymine derivative were isolated and characterized spectrophotometrically. Protamine titration of the DNA–mustard gas product indicated that primary phosphoryl groups had been esterified. It is difficult to reconcile this conclusion with a previous finding that phosphate esterification did not occur. The latter result was obtained by measuring the release of hydrogen ion during reaction.

Introduction

If it is true that the biological effects of the mustards and other alkylating agents are due to their reaction with the deoxyribonucleic acid (DNA) of the cell, then a knowledge of the chemical reactions that occur between these agents and DNA would be desirable. On the assumption that this explanation of the biological effects of the alkylating agents is valid or at the least, reasonable, we have previously studied the reaction of mustard gas, first with the free purines and pyrimidines (1), and second with the purine portion of the DNA molecule (2). In these two papers the pertinent literature has been reviewed.

Very recently Brookes and Lawley (3) have reported the results of a study of the reaction between S^{35}-labelled mustard gas and DNA in which the molar ratio of mustard gas to DNA phosphorus was 1:1000. This low ratio was considered to approach that which would be encountered intracellularly. They found that only the guanine of DNA was attacked (at the 7-position). This finding may render our previous work, and that of earlier authors, unmeaningful in a biological sense because rather large amounts of alkylating agents had been used. The results to be presented, which deal mainly with the chemical reactions of mustard gas with the non-purine portion of DNA, may serve more to document the various reactions of mustard gas than to explain the biological effects of this agent.

Methods

Reaction of DNA with Mustard Gas and Fractionation of the Product

Details of the method have been reported previously (2). Briefly, the

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Issued as PCC No. D50-93-25-07 and DRML Report No. 220-3.

mustard gas and DNA in the desired molar ratio* were allowed to react at pH 7.4. The reaction mixture was dialyzed thoroughly against cold water, and then at 37° against a hydrochloric acid solution, pH 1.6, which releases the purines quantitatively (4). The material left in the sac is known as apurinic acid (APA). The purines were adsorbed from solution by passage through a strongly acidic cation exchange column and released by 0.1 *N* ammonium hydroxide. The apurinic acid fraction remaining in the dialysis sac was neutralized to pH 7 and dialyzed against cold water.

Hydrolysis of Apurinic Acid

The neutralized and dialyzed sac contents were filtered to remove a small amount of biuret positive material, vacuum distilled at 40° to near dryness, and the drying was completed in an evacuated desiccator over calcium chloride. The dry residue from 100 mg of original DNA was dissolved in either 1.5 ml of 70% perchloric acid or 5 ml of 85–90% formic acid and hydrolyzed.

(*A*) *Perchloric Acid*

The perchloric acid solution was heated at 100° for 60 minutes, and then was diluted 10-fold with water. Carbonaceous material was removed by centrifugation, and was washed with water. The combined washings and supernatant were neutralized with potassium hydroxide and left in the cold to allow potassium perchlorate to precipitate. The potassium perchlorate was centrifuged off, washed with small amounts of cold water, and the washings and supernatant were combined. Part of this solution was passed through a strongly acidic cation exchange column (Dowex-50) in the hydrogen form, in order to separate cytosine and its derivatives from thymine and its derivatives. After the column was washed with water until no more ultraviolet light (260 mμ) absorbing material was eluted, the cytosine and its derivatives retained by the column were eluted with 0.2 *N* ammonium hydroxide. The separate pyrimidine solutions were concentrated by vacuum distillation at 40°. The other part of the neutralized hydrolyzate from which potassium perchlorate had been removed was distilled to dryness under vacuum at 40°. The residue which still contained much potassium perchlorate was extracted with small amounts of water.

(*B*) *Formic Acid*

One-half milliliter samples of the formic acid solution were heated in sealed glass tubes at 270° for 45 minutes. The contents of two tubes were pooled and distilled to dryness. The residue was dissolved in 5 ml of water, distilled to dryness, and finally redissolved in water. The aqueous solution was passed through a cation exchange column (Dowex-50) to separate the cytosine fraction from the thymine fraction as described under the perchloric acid treatment. These fractions were prepared solely for the paper chromatographic estimation of the amounts of unreacted cytosine and thymine.

*A molar ratio, mustard gas:DNA phosphorus (H/P ratio) of 2.3, was employed except in one instance which is noted.

Paper Chromatography

The descending method was used with Whatman No. 1 paper or Whatman No. 3 paper to which a small wick of No. 1 paper had been sewn to retard the flow. The solvents were (A) butanol–water (86:14 (v/v)) and (B) isopropyl-alcohol – hydrochloric acid (isopropyl alcohol 170 ml, concentrated hydrochloric acid 41 ml, water to make 250 ml). Solvent (B) was used in the quantitative estimation of cytosine because a fluorescent material, closely associated with cytosine, was seen when aqueous butanol was used. The quantitative technique followed was that described previously (2).

Titration of DNA with Protamine

The method described by Reiner and Zamenhof using salmine sulphate as the protamine was followed (5). Both the mustard-treated DNA and control DNA were dialyzed thoroughly in the cold before titrating them. The rationale and appropriate data are as follows. Eosin is used as an indicator. Protamine combines through its positively charged arginine groups with the negatively charged phosphate groups of DNA to form an insoluble complex. The first excess of protamine combines with the eosin and quenches its fluorescence. One millimole of arginine is contained in 0.255 g of salmine sulphate and 1 millimole of phosphorus is contained in 0.327 g of nucleic acid (5). The ratio of these two weights is 0.78.

Sulphur Analysis

The method has been described previously (1).

Spectrophotometry

A Beckman DU spectrophotometer with a DR recording attachment was used. The buffered solutions utilized were 0.1 *M* borate, pH 9.3, and a pH 7 buffer prepared by Beckman Instruments, Ltd.

Results

The extent of the reaction of mustard gas with DNA as a whole and with its purine and non-purine components was estimated by measuring the sulphur content of these materials. The figures obtained are shown in Table I and it

TABLE I

The reaction of mustard gas with DNA based on sulphur analysis

	H/P = 2.3		H/P = 0.29	
	(1)	(2)	(1)	(2)
Percentage of mustard reacting with DNA	19	21	36	27
Percentage of reacted mustard bound to purines*	37	36	53	50
Percentage of reacted mustard bound to APA	28	21	26	20
Percentage of reacted mustard released from DNA by mild acid treatment and not bound to purines or APA†	33	—	—	—

*Sulphur component in purine fraction which was retained by, and subsequently eluted from, a cation exchange column.

†Sulphur component in purine fraction which was not retained by a cation exchange column.

will be seen in the first column that 98% of the sulphur bound to DNA was accounted for when all fractions were analyzed. When the purine fraction was passed through a column of strongly acidic cation exchange resin, a portion of the sulphur-containing material was not retained by the column and this fraction did not absorb ultraviolet light. Thus, as shown in the table, part of the sulphur in the purine fraction is bound to purine and part is not.

In order to assess whether DNA phosphate was esterified after reaction with mustard gas, the product was titrated with protamine. The result shown in Table II suggests that DNA phosphate has been esterified.

TABLE II

The titration of mustard-treated DNA with protamine

	Weight ratio, $\frac{\text{salmine sulphate}}{\text{DNA}}$	
	Dialyzed DNA	Dialyzed, mustard-treated DNA (H/P = 2.3)
Theoretical	0.78	—
Observed	0.80	0.57

The APA fraction from reacted DNA was hydrolyzed with perchloric acid or formic acid and the resulting free pyrimidines were chromatographed on paper before and after separating them by means of a cation exchange resin into a thymine and a cytosine fraction. Figure 1 is a drawing of a chromatogram of the pyrimidines obtained after perchloric acid hydrolysis. The cytosine and thymine content of the separated fractions was measured by

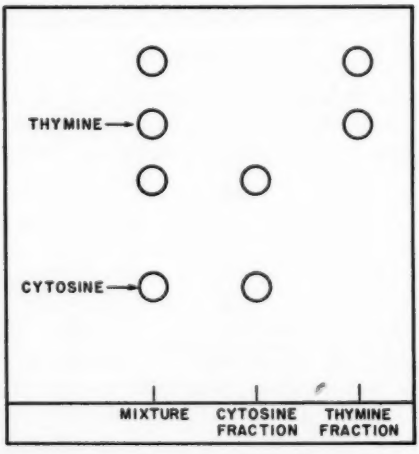

FIG. 1. Drawing of chromatogram of pyrimidines obtained from DNA which had been reacted with mustard gas. Solvent was butanol: water, 86:14 (v/v), run on Whatman No. 1 paper for 16.5 hours.

quantitative paper chromatography and the values obtained are shown in Table III. Finally, the ultraviolet absorption spectra of the cytosine and

TABLE III

The cytosine and thymine content of DNA after reaction with mustard gas (H/P = 2.3)

	Total extinction* of pyrimidine		
	Control DNA	Mustard-gas-treated DNA	% of control value
Perchloric acid hydrolysis			
Cytosine	958	862	90
Thymine	823	751	91
Formic acid hydrolysis			
Cytosine	955	771	81
Thymine	916	824	90

*Measured in 1 *N* hydrochloric acid at 265 mμ for thymine and at 275 mμ for cytosine. Total extinction calculated for the amount of pyrimidine derived from original 200 ml of 0.1% DNA.

thymine derivatives obtained by elution from paper chromatograms are presented in Fig. 2 and Fig. 3 respectively.

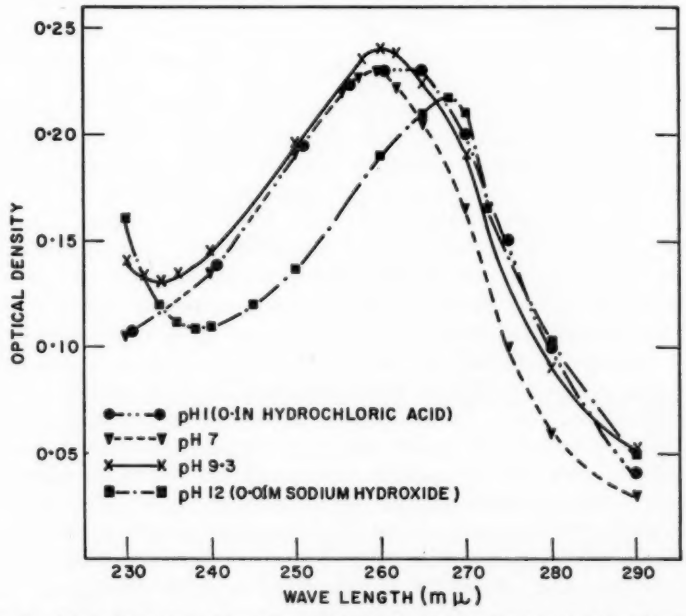

FIG. 2. Ultraviolet absorption spectra of the cytosine derivative obtained from DNA after reaction with mustard gas.

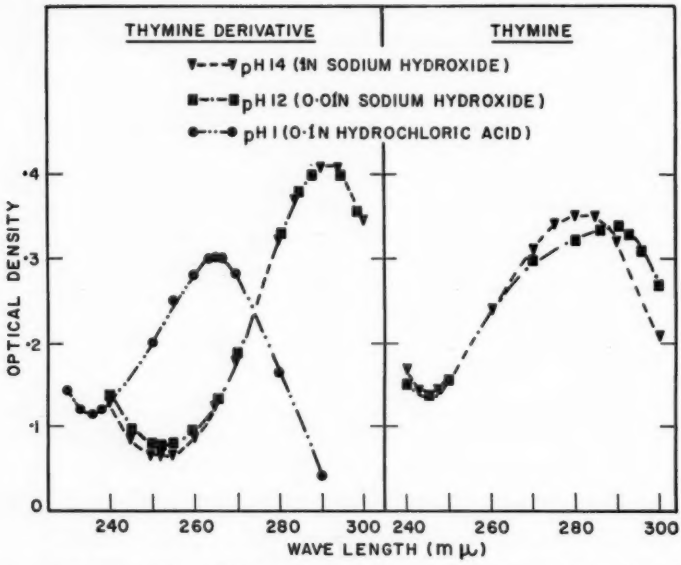

FIG. 3. Ultraviolet absorption spectra of thymine and the thymine derivative obtained from DNA after treatment with mustard gas.

Discussion

The data in Table I show that mustard gas reacts with DNA in three distinct ways: with the purines, with the APA moiety to form stable compounds; with some unknown portion of the DNA molecule to give a material which becomes dializable after mild acid treatment. The site of attachment of this sulphur component prior to dialysis at pH 1.6, whether to purine or to the APA moiety remains unknown. The possible sites of reaction of mustard gas with the APA moiety are the pyrimidines or the phosphate groups. It was shown previously that 2 moles of hydrogen ion per mole of mustard gas was released during reaction with DNA (2). This was taken as evidence that the mustard did not react with primary phosphoryl groups since, at pH 7.4, reaction with primary phosphate would yield sodium chloride and not hydrogen chloride. Stacey *et al.* (6) had shown previously, by this technique, that phenylalanine mustard does react with the primary phosphoryl groups of DNA and we were able to confirm this result (2). Since an independent assessment of the possible reaction of mustard gas with DNA-phosphate seemed desirable the mustard-treated DNA was titrated with protamine. The result (Table II) indicates that 29% of the primary phosphate has been esterified by the mustard gas. It is difficult to reconcile this result with the previous one unless it is considered that the protamine titration is not a true indication of the number of primary phosphoryl groups in DNA after reaction

with mustard gas. An error in technique would appear to be ruled out by the titration result obtained with the control DNA.

When the APA was hydrolyzed with perchloric acid and then chromatographed on paper, two substances besides cytosine and thymine were observed. The identity of the parent pyrimidine of each of these substances was revealed by passing the hydrolyzate through a cation exchange column, since cytosine and its derivatives are retained but thymine and its derivatives are not. When these fractions were chromatographed separately it was seen that both cytosine and thymine had reacted with mustard gas (Fig. 1). Quantitative paper chromatographic analysis indicated the extent of these reactions, namely, 10%–19% for cytosine and 9%–10% for thymine (Table III). From the data in Table I (H/P = 2.3), it can be calculated that 0.115 mole of mustard has reacted with 0.5 mole of pyrimidine, or 23% of the pyrimidine has reacted. This is in excellent agreement with the chromatographic findings and suggests that the mustard bound to the APA fraction is associated only with the pyrimidines.

The spectral curves for the cytosine derivative (Fig. 2) indicate that a hydroxyl group is present because of the ionization between pH 9.3 and pH 12. There is also a group ionizing between pH 1 and pH 7. In order for cytosine to be alkylated and still show these two ionizable groups, the alkylation must have occurred at the amino group yielding a secondary amine, or at the N_3 position thereby converting the amino into an imino group. The spectral curves obtained for the thymine derivative at pH 12 and pH 14 are considered to be the same (Fig. 3). The slight difference between the two curves in the range 240 mμ – 260 mμ is thought to be due to impurity. Certainly a wave-length shift in the maximum wave length is not apparent as it is for thymine (Fig. 3). Thus one of the hydroxyls of the thymine derivative has been alkylated or converted permanently to the keto form through alkylation of a ring nitrogen.

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HYDROLYSIS OF CONJUGATED ESTROGEN FRACTIONS IN HUMAN PREGNANCY URINE[1]

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Abstract

The release of six estrogen fractions from conjugation in human pregnancy urines has been studied using various hydrolytic methods. The estrogens concerned were estrone, estradiol-17β (estradiol), 2-methoxyestrone, 16-epiestriol, and a ring D ketolic fraction (mainly 16α-hydroxyestrone). Considerable amounts of urinary estrone and ring D ketolic estrogens may be conjugated in a non-glucuronide form. In these cases an enzyme preparation containing β-glucuronidase and sulphatase, prepared from the digestive juice of the snail *Helix pomatia*, proved to be superior to β-glucuronidase enzymes of bacterial or mammalian liver origin. Conventional hot acid hydrolysis yielded levels of estrone, estradiol, estriol, and 16-epiestriol which agreed fairly well with those obtained following snail enzyme hydrolysis. In some urines, hot acid treatment was not suitable for hydrolysis of conjugated 2-methoxyestrone. Optimum hydrolytic conditions for both normal and diabetic pregnancy urines were realized by incubating for 24 hours with 500 units of the snail β-glucuronidase and 250 units of sulphatase/ml of urine at pH 5.2 and 37–38° C.

Introduction

The problem of hydrolyzing urinary estrogen conjugates prior to estrogen analysis is not a new one. Within recent years, however, new problems have arisen due to the discovery of labile ring D ketolic compounds (e.g. 16α-hydroxyestrone) which cannot be treated by conventional hot acid hydrolysis without destruction (1). Moreover, in urines containing glucose, destruction of even the relatively stable 'classical' estrogens, namely, estrone, estradiol-17β (estradiol), and estriol, has been observed during hot acid treatment (2, 3). Since the ring D ketolic estrogens seem to be of considerable importance in the general scheme of estrogen metabolism (4), and since estrogen patterns in diabetic pregnancy urines may be of interest (5), mild methods of hydrolysis, such as incubation with β-glucuronidase and sulphatase preparations, have become increasingly necessary.

A number of investigators have reported upon the efficiency of enzyme hydrolysis as compared with conventional hot acid treatment (2, 6–10). Such studies have also yielded some indirect information regarding the mode of conjugation of certain of the urinary estrogens. However, little is known about the hydrolytic release of the more recently discovered metabolites such as 2-methoxyestrone, 16-epiestriol, and 16α-hydroxyestrone. Knowledge of this sort might lead to a clearer understanding of the over-all metabolism of estrogen secreted by the human subject.

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The present report concerns the use of various hydrolytic techniques for the cleavage of conjugated estrogen metabolites in human pregnancy urine. The compounds investigated include estrone, estradiol, estriol, 2-methoxyestrone, 16-epiestriol, and a fraction referred to as 'ring D ketolic', this latter apparently consisting mainly of 16α-hydroxyestrone but also containing 16-ketoestradiol-17β and probably 16β-hydroxyestrone. Particular attention has been given, in the present study, to various aspects of the action of an enzyme preparation from the digestive juice of the snail *Helix pomatia*.

Material and Methods

Urine Collection

Complete 24-hour specimens were collected without preservative from some 25 women, both normal and diabetic, during the last 20 weeks of pregnancy. Following the final collection each urine was despatched to the laboratory without delay and hydrolysis was immediately begun. Where delays were necessary between the termination of enzyme incubation and subsequent analysis the urine was frozen after incubation until required.

Chemicals

Organic solvents and other reagents were Reagent Grade and were purified where necessary by published procedures (11, 12).

Enzyme Preparations

Bacterial β-glucuronidase, Sigma Chemical Co.; mammalian liver β-glucuronidase (Ketodase), Warner Chilcott Laboratories Inc.; molluscan β-glucuronidase plus sulphatase (Glusulase), prepared from the snail *Helix pomatia*, Endo Laboratories Inc.; molluscan β-glucuronidase plus sulphatase, prepared from the common limpet *Patella vulgata;* powder B of Dodgson and Spencer (13).

The unit of activity of each preparation was based on its β-glucuronidase content and is defined as the amount of enzyme liberating 1 μg of phenolphthalein from a 0.001 *M* solution of phenolphthalein glucuronide in 1 hour at 37° C.* The pH of incubation was 6.5 for the bacterial preparation, 5.0 for the liver enzyme, 5.2 for the snail preparation, and 4.6 for the limpet material.

Hydrolysis of the Estrogen Conjugates

For enzyme hydrolysis, 10- or 20-ml volumes of urine were incubated, in duplicate, with one or the other of the enzyme preparations at varying enzyme concentrations, pH values, or for varying times, at 37–38° C. Adjustment of pH for the various enzymes was made as described earlier (15). In most cases penicillin (2000 units/ml of urine) was added prior to incubation (16).

Where hot acid hydrolysis was performed 10- or 20-ml volumes of urine, diluted 1:10 with distilled water, were hydrolyzed by boiling for 1 hour under

*The unit of activity of the bacterial preparation should be modified by indicating that assay was performed in the presence of chloroform. This exerts an 'activating' effect (14) and the unit is less than is the case in the absence of chloroform when phenolphthalein glucuronide is the substrate.

reflux with 15 volumes % of HCl (11); this acid concentration referring to diluted urine.

Splitting of residual labile estrogen conjugates (e.g. sulphates) following enzyme hydrolysis was performed by adjusting the urine (from which free estrogens had already been extracted) to pH 1 with HCl and continuously extracting with ether in an all-glass apparatus for 48 hours at room temperature (17). Estrogen thus released was considered to have arisen from labile non-glucuronide conjugates which were not hydrolyzed by the enzyme preparations.

Extraction and Analysis of the Estrogens

In all cases incubated urine was diluted to 100 ml with water prior to extraction. In experiments where only estrone, estradiol, and estriol were to be measured, hydrolysis was followed by the modified method of Bauld (3, 18). For the analysis of six estrogen fractions, including the labile ring D ketolic compounds, the method of Givner *et al.* (12) was employed, modified by omitting the second Girard separation and saponification of the estradiol and 2-methoxyestrone fractions following partition chromatography, and by reducing solvent and reagent volumes where necessary. Where the ring D ketolic fraction was not to be measured, as in analyses following hot acid hydrolysis, the initial NaOH–bicarbonate treatment of the ether extract was employed according to Bauld (11), followed by the Girard separation and subsequent steps of the method of Givner *et al.* (12).

Recovery Experiments

For the purpose of checking the accuracy of the analytical methods, known amounts of pure estrogens (5–25 μg depending on the compound concerned) were added to 10- or 20-ml volumes of non-pregnancy (male or female) urine which had been hydrolyzed either by the enzymic or hot acid method. These urines were then analyzed by the techniques described above. Recoveries were also measured of estrogens (excluding the ring D ketolic compounds) added to urine prior to hot acid hydrolysis. In studying the recovery of the ring D ketolic estrogens 16α-hydroxyestrone was normally employed as the representative compound but some experiments were also carried out using 16-keto-estradiol-17β.

Results

Accuracy of the Methods

Table I deals with the accuracy of the analytical procedures. Recovery of the various estrogens was not influenced by the enzyme preparation used. With the possible exception of 16-epiestriol, recovery of the pure estrogens was also independent of whether these were added to enzyme- or acid-hydrolyzed urine. Recovery of added estrone, estradiol, estriol, and 2-methoxyestrone was the same whether these steroids were added before or after hot acid hydrolysis. Good agreement, considering the different techniques involved, existed between the methods of Bauld (11) and Givner *et al.* (12) with respect to recovery of estrone, estradiol, and estriol.

TABLE I

Percentage recoveries of estrogens added to non-pregnancy urines
(Mean values are given ± standard deviation or with ranges in parentheses)

Analytical method	Hydrolytic method	Estrogen added					
		Estrone	Estradiol	Estriol	2-Methoxy-estrone	16-epiEstriol	16α-Hydroxyestrone or 16-ketoestradiol-17β
Givner *et al.* (12)	Enzyme*	85 ± 3.7 *n* = 22§	80 ± 5.6 *n* = 14	75 ± 5.0 *n* = 23	72 ± 6.0 *n* = 12	75 ± 5.8 *n* = 14	64 ± 4.7 *n* = 16
Givner *et al.* (12)	Hot acid†	90(85–96) *n* = 4	81(79–85) *n* = 4	71(64–75) *n* = 4	76(74–81) *n* = 4	84(76–90) *n* = 4	—
Givner *et al.* (12)	Hot acid‡	86(83–87) *n* = 3	79(77–83) *n* = 3	72(67–78) *n* = 3	77(70–88) *n* = 3	78(75–83) *n* = 3	—
Bauld (11)	Hot acid‡	92 ± 2.1 *n* = 10	83 ± 2.5 *n* = 10	80 ± 3.2 *n* = 12	—	—	—

*Employing 20 units bacterial enzyme/ml at pH 6.5; 300 units liver enzyme, pH 5.0; 500 units snail preparation, pH 5.2; or 750 units limpet preparation, pH 4.6.
†Estrogens added after hydrolysis.
‡Estrogens added before hydrolysis.
§*n* = number of duplicate analyses.

Hydrolysis by Various Methods

The effect of varying incubation time on the enzymic release of the three main estrogen fractions, estriol, estrone, and ring D ketolic estrogens, is shown in Figs. 1, 2, and 3. Within 48 hours of incubation the bacterial, limpet, and

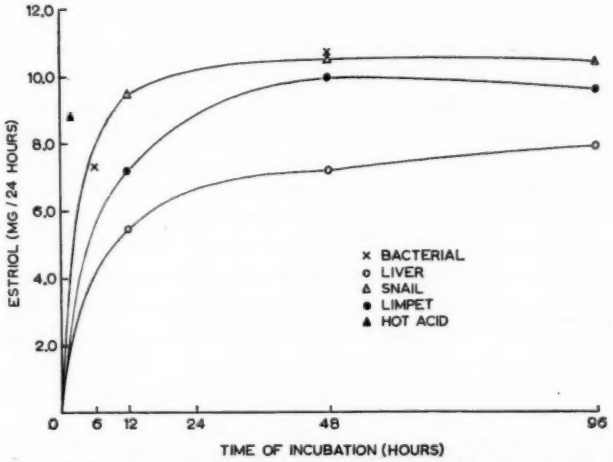

FIG. 1. Effect of incubation time on the liberation of estriol in normal human pregnancy urine by β-glucuronidase-containing preparations of bacterial, mammalian liver, and molluscan origin. Conditions of hydrolysis as in Table I and in text.

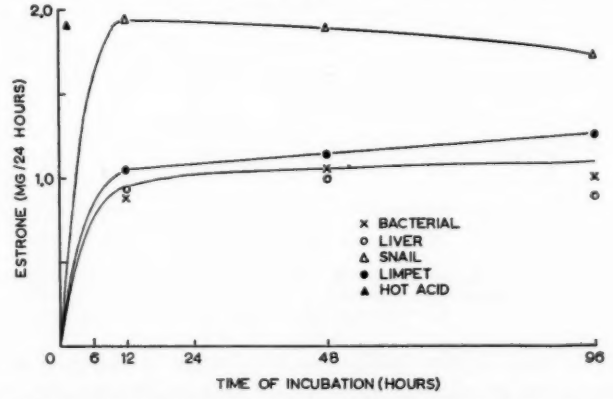

FIG. 2. Effect of incubation time on the liberation of estrone in normal human pregnancy urine by β-glucuronidase-containing preparations of bacterial, mammalian liver, and molluscan origin. Conditions of hydrolysis as in Table I and in text.

snail preparations gave rise to rather higher estriol levels than did conventional hot acid hydrolysis. Continuous ether extraction following 96 hours of snail enzyme activity released only an additional 3% of estriol while a further 2%,

4%, and 10% was released by this procedure after incubation with bacterial (48 hours), limpet (96 hours), and mammalian liver (96 hours) enzymes, respectively. It was apparent from this that little estriol was present as a labile conjugate. The difference between the liver enzyme and the other preparations was considered to be due to slower hydrolysis of estriol glucuronide by the former enzyme (19).

In a second normal pregnancy urine, maximum hydrolysis of estrone conjugates was attained within 12 hours with all four enzyme preparations (Fig. 2). However, this maximum was much greater in the case of the snail preparation, the value being the same as that obtained by hot acid hydrolysis. Continuous ether extraction following incubation with the other three enzyme preparations released sufficient estrone to account for the observed differences, suggesting considerable (40–50%) conjugation of the steroid in a non-glucuronide form in this particular urine and perhaps even a labile conjugate, other than sulphate, split by the snail preparation but not by the sulphatase-containing limpet one. Very similar results were obtained for the ring D ketolic fraction in the same urine (Fig. 3). Continuous ether extraction subsequent to enzyme hydrolysis resulted in no further measurable release of ring D ketolic estrogens,

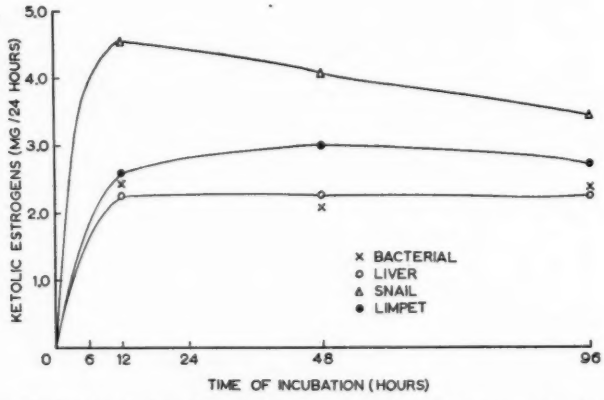

FIG. 3. Effect of incubation time on the liberation of ketolic estrogens in normal human pregnancy urine by β-glucuronidase-containing preparations of bacterial, mammalian liver, and molluscan origin. Conditions of hydrolysis as in Table I and in text.

presumably because of the labile nature of these compounds under acidic conditions. It can be seen in Fig. 3 that a marked decrease occurred in the ring D ketolic fraction when incubated with the snail preparation between 12 and 96 hours. This was also true of the estrone fraction in the same urine (Fig. 2). Antibiotics were not used in these particular incubations. Even in the presence of penicillin, however, a similar fall occasionally occurred in the ring D ketolic fraction.

Table II compares the efficiencies of different hydrolytic methods for the

TABLE II

Liberation of estrone (O), estradiol (D), and estriol (T) in a normal human pregnancy urine by different hydrolytic methods (Results expressed as mg/24 hours)

Hydrolytic* method	O + D + T liberated	O + D + T liberated by continuous extraction after enzyme hydrolysis	Total	Total, as % of snail enzyme + continuous extraction
Hot acid	11.6	—	11.6	87
Bacterial enzyme	12.7	0.73	13.4	101
Mammalian liver enzyme	10.0	1.33	11.3	85
Limpet enzyme	11.8	0.59	12.4	93
Snail enzyme	13.0	0.34	13.3	100

*Enzyme concentrations and conditions of pH as in Table I; times of hydrolysis were bacterial, 48 hours; mammalian liver, 96 hours; snail, 96 hours; and limpet, 96 hours.

release of estrone, estradiol, and estriol in a normal pregnancy urine. All four enzyme preparations used yielded results which compared at least favorably with those obtained by hot acid hydrolysis. When five normal pregnancy urines were separately hydrolyzed with hot acid, snail preparation, and bacterial preparation, and also analyzed for estrone, the results (Table III) suggested very variable amounts of this steroid to be present as non-glucuronide ('sulphate') conjugates.

TABLE III

Liberation of estrone in normal human pregnancy urines by different hydrolytic methods (Results expressed as mg/24 hours)

Hydrolytic* method	Normal pregnancy urines from different subjects				
	1†	2†	3	4	5
Hot acid	2.4	1.9	0.59	0.69	0.77
Snail enzyme	2.4	1.9	0.62	0.69	0.76
Bacterial enzyme	1.6	1.1	0.55	0.65	0.59
Bacterial, as % of hot acid or snail enzyme	67	58	90	94	77

*Enzyme concentrations and conditions of pH as in Table I; time of hydrolysis for enzymes, 24 hours.
†Estrone liberated by continuous ether extraction following action of bacterial enzyme accounted for difference between the latter and the other hydrolytic methods.

Effect of pH on the Activity of the Snail Preparation

Aliquots of a normal pregnancy urine were incubated for 24 hours with amounts of the snail preparation corresponding to 500 units of β-glucuronidase activity/ml of urine at pH values of 4.0–6.5 at pH unit intervals of 0.5. Release of the six estrogen fractions measured did not vary significantly over the pH range studied except in the case of estriol which showed a considerable decrease at 6.5 (70% of the level obtained between pH 4.0 and pH 6.0).

Effect of Variation of the Snail Enzyme Concentration

Aliquots of five urines were incubated with varying concentrations of the snail preparation (100–1000 units of β-glucuronidase/ml of urine). Table IV contains the results obtained. In only three of the urines was measurable 2-methoxyestrone found (0.19–0.66 mg/24 hours). It was observed that 100 units of activity was sufficient for maximum hydrolysis of conjugated estrone, estradiol, 16-epiestriol, and the ring D ketolic fraction in 24 hours, but not for 2-methoxyestrone or estriol. Five hundred units appeared to offer a safe compromise since 1000 units did not result in any consistent increase in hydrolysis.

Effect of Variation of Incubation Time on Activity of the Snail Preparation

Aliquots of five pregnancy urines were incubated with the snail enzyme preparation for varying times from 6 to 96 hours. In only two of these was measurable 2-methoxyestrone found (0.19–0.30 mg/24 hours). The results in Table V show considerable hydrolysis to have occurred in 6 hours for all fractions, with 2-methoxyestrone and estriol lower than the others. In 16 hours approximately, maximum release of all fractions was apparent although

TABLE IV

Liberation of six estrogen fractions in human pregnancy urines* by different concentrations of snail enzyme preparation (Mean results (with ranges) expressed as percentage of values obtained with 500 units of enzyme/ml for each urine)

Estrogen fraction	Units of β-glucuronidase/ml of urine				
	100	250	500	750	1000
Estrone	104	100 (94–105)	100	101 (100–103)	99 (98–100)
Estradiol	92	97 (87–100)	100	91 (87–104)	84 (72–96)
Estriol	77	95 (87–101)	100	101 (91–110)	104 (103–105)
2-Methoxyestrone	81	89 (82–99)	100	97 (90–100)	83
16-epiEstriol	108	101 (96–108)	100	97 (92–101)	96 (92–100)
Ring D ketolic fraction	102	104 (98–112)	100	109 (101–118)	100 (93–102)

*Two normal and three diabetic urines were employed with incubation periods of 24 hours at pH 5.2. In the case of all the fractions at the 100-unit level and or 2-methoxyestrone at the 1000-unit level only one urine was analyzed.

96 hours' incubation did result, in some urines, in a further increase in the ring D ketolic fraction. However, as has already been mentioned, decreased ring D ketolic levels could also occur, even in the presence of antibiotics. For these reasons an incubation period of 24 hours was considered to be most suitable. The rapid release of estriol by this snail preparation (Fig. 4) was particularly noteworthy in view of the slower hydrolysis by other enzyme preparations (2, 19).

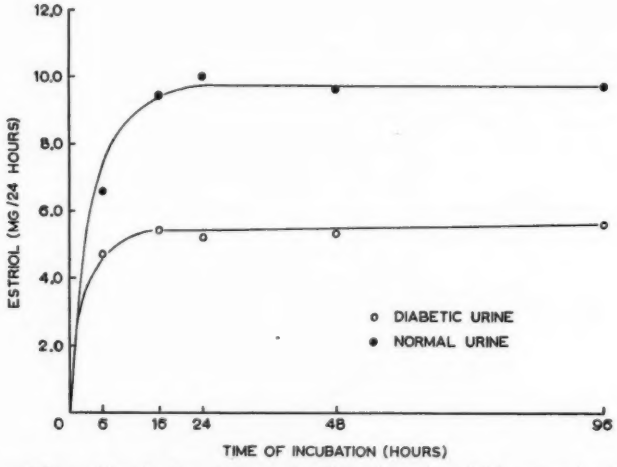

FIG. 4. Effect of incubation time on the liberation of estriol in normal and diabetic pregnancy urines by the snail enzyme preparation. Conditions of hydrolysis as in Table I for the snail preparation.

Comparison of Hot Acid with Snail Enzyme as Hydrolytic Methods

Aliquots of pregnancy urines (both normal and diabetic) were incubated for 24 hours at 37° C and pH 5.2 with a concentration of the snail preparation equivalent to 500 units of β-glucuronidase/ml of urine. Five estrogen fractions (excluding the ring D ketolic estrogens) were measured, and their amounts compared with those liberated in the same urines by hot acid hydrolysis after 1:10 dilution. The results for four fractions are shown in Table VI. Good agreement was found between the two hydrolytic methods for the release of estrone, estradiol, and estriol with the results slightly, although not significantly, in favor of enzyme hydrolysis. The converse was true for 16-epiestriol although again there was no significant difference. These findings are in agreement with those of other workers who used limpet enzyme preparations (2, 20).

In four urines the 2-methoxyestrone released by acid amounted to 60–87% (mean, 77%) of that obtained by snail enzyme hydrolysis. In two other urines no measurable 2-methoxyestrone was found after hot acid hydrolysis although 0.2 and 0.3 mg of this steroid/24 hours was measured after incubation with the snail preparation. These results are difficult to interpret since preliminary

TABLE V

Liberation of six estrogen fractions in human pregnancy urines* when incubated for varying times with the snail enzyme preparation (Mean results (with ranges) expressed as percentages of values obtained after 24 hours for each urine)

Estrogen fraction	Time of hydrolysis (hours)				
	6	16	24	48	96
Estrone	95 (89–103)	98 (93–102)	100	98 (93–105)	95 (89–104)
Estradiol	92 (91–93)	99 (88–109)	100	96 (91–104)	111 (109–112)
Estriol	78 (66–92)	97 (92–104)	100	101 (96–107)	103 (97–109)
2-Methoxyestrone	75	100 (97–103)	100	96 (82–109)	97 (82–111)
16-epiEstriol	99 (92–103)	103 (97–106)	100	99 (94–100)	104 (102–106)
Ring D ketolic fraction	90 (88–93)	95 (88–100)	100	97 (91–100)	96 (76–115)

*Three normal and two diabetic urines were employed with enzyme concentrations of 500 units/ml of urine at pH 5.2. In the case of 2-methoxyestrone at 6-hour incubation time only one urine was analyzed.

TABLE VI

Comparison of hot acid and snail enzyme hydrolysis
(Results expressed as acid/enzyme×100; mean values are given with number of duplicate analyses in parentheses)

Estrone	Estradiol	Estriol	16-epiEstriol	Total*
95±7.6† (15)	89±17.0† (9)	89±6.5† (9)	109±16.7† (10)	90±6.7† (8)

*Total refers to the addition of the four estrogen fractions in the table.
†Standard deviation.

experiments in this laboratory showed free 2-methoxyestrone to be stable to hot acid hydrolysis in the presence of urine. In some urines, however, considerable 'background' chromogen contamination was produced in the 2-methoxyestrone fraction by acid hydrolysis. This could be particularly troublesome when very low levels of this steroid were being measured. This observation, together with the possibility that the effect of acid on free and conjugated 2-methoxyestrone may not be the same, could perhaps account for the above results.

Discussion

Since recovery of the various estrogens added to hydrolyzed urine was independent of the hydrolytic method employed, the difference in estrogen levels found after incubation of certain urines with enzyme preparations from different sources was probably a true reflection of a variation in the ability of these enzymes to split urinary estrogen conjugates under the experimental conditions. The amounts of bacterial, mammalian, and limpet enzymes used in the present study, although not necessarily ideal, have been shown by others to yield optimum hydrolysis of estrogen conjugates given sufficient time for action to occur (2, 21, 22). It is improbable that higher concentrations of bacterial or mammalian preparations could have increased the extent of hydrolysis of conjugated estrone in those urines where the snail preparation released significantly more of this steroid, since the difference was due to labile (non-glucuronide) conjugates. There is no good evidence that enzymes capable of splitting the latter are to be found in either the bacterial or mammalian liver preparations. In the case of the limpet preparation, however, one would have expected to observe a greater release of estrone provided that the non-glucuronide conjugate was sulphate. This is discussed further below. These observations, together with the facility possessed by the snail preparation for releasing estriol and ring D ketolic estrogen metabolites, showed that this enzyme mixture was a very suitable one for the present purpose. Slaunwhite and Sandberg (23) have reported the snail preparation to be better than enzymes of bacterial or mammalian liver origin for the hydrolysis of conjugated estrogens in urine. These workers obtained maximum hydrolysis by incubating urine for 96 hours at pH 5.5 and 37° C with 300 units/ml of the snail β-glucuronidase together with sulphatase. These results were based on the measurement of

total radioactivity released in the urine following the administration of C^{14}-labelled estrogen to human subjects and therefore provided no information regarding the effect of this hydrolytic procedure on individual metabolites.

It was established by Brown and Blair (2) that, provided urine was first diluted 1:10 with water, no destruction of added free estrone, estradiol, or estriol occurred during hot acid hydrolysis. Moreover, this method of hydrolysis was shown to yield levels of these three estrogens which compared very closely with those obtained by incubating with the limpet enzyme preparation. Thus it appeared likely that the limpet enzymes were capable of affecting more or less complete release of the metabolites in question. By the same token it could be suggested from the results of the present study that this is also true of the snail preparation and that it extends to include 16-epiestriol. A few results obtained showed that the snail preparation could be significantly more active than the limpet one in releasing estrone and ring D ketolic estrogens. In the case of estrone this was apparently due to a failure to hydrolyze non-glucuronide conjugates since continuous ether extraction at pH 1 accounted for the difference. This finding was surprising in view of the sulphatase present in the limpet preparation, which enzyme would be expected to split estrone sulphate. It is not clear at present whether a non-glucuronide conjugate, other than sulphate, could have existed in this urine and might have been hydrolyzed by some other enzyme present in the snail preparation but absent from the limpet material. Bloch and Gibree (10) reported much higher (70–100%) levels of estrone, estradiol, and estriol following incubation with 1500 units of snail β-glucuronidase, and at least 500 units of sulphatase/ml of urine for 24 hours at pH 5.2 and 39° C, than after hot acid hydrolysis. However, these workers used non-pregnancy urines of very low estrogen titer without dilution prior to acid hydrolysis; these conditions were known to be quite destructive (2). Jayle *et al.* (24) used 750–1500 units of snail β-glucuronidase and at least 500 units of sulphatase/ml of urine for the hydrolysis of conjugated estrone, estradiol, and estriol and obtained better results than with either bacterial β-glucuronidase or hot acid. In the present study, each 500 units of snail β-glucuronidase was accompanied by approximately 250 units of sulphatase activity.

Comparison of hot acid and enzyme hydrolysis has not been possible for conjugated ring D ketolic estrogens or 2-methoxyestrone due to the adverse effect of hot acid on these fractions. Moreover, the picture is further complicated for 2-methoxyestrone by the number of pregnancy urines in which measurable amounts of this steroid do not seem to occur (25). Thus no definite conclusion has been reached regarding the quantitative release of these estrogens by the snail preparation. However, it does seem likely that the hydrolytic conditions finally chosen are capable of yielding approximately maximum release of the six estrogen fractions concerned. In none of the experiments performed was there any marked difference in hydrolytic efficiency by the snail preparation whether normal or diabetic urines were studied. This showed that

the enzymes were not adversely affected by the usual constituents of diabetic urine.

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BIOCHEMICAL RESPONSE OF THE PERITONEAL MUCOSA TO THE IRRITANT ACTION OF FINELY DIVIDED SILICA AND OTHER DUSTS[1]

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Abstract

Repeated injection of fluid suspensions of finely divided silica (quartz) into the peritoneal cavity of the rat gave rise to an increase in the concentration of lactate and α-amino nitrogen and to a decrease in the concentration of glucose in the peritoneal exudate. The silica greatly diminished the selective 'permeability' of the peritoneal membranes as evidenced by the retention of injected phenol red in the peritoneal fluid. Silica increased the concentration of trypsin inhibitor in the serum. None of these changes occurred upon repeated injection of suspensions of finely divided carbon or carborundum, or of silicic acid sol. The observations throw light on the fibrogenic action of quartz dust in silicosis.

Finely divided silica, when inhaled as dust or injected as a suspension in a liquid into the tissues or body cavities, induces an inflammatory reaction of the tissue with the formation of collagenous nodules. The nodules consist of concentric layers of collagen with the silica particles embedded in them. The mode of formation and the histological structure of the nodules have been described by many workers (1, 2, 3) but the nature of the biochemical response of the tissue is not understood.

Miller and Sayers (1) have studied the histological response of the peritoneal mucosa to finely divided dusts of various kinds. They observed that only the dusts containing silica induced formation of nodules of the type that occurs in the silicotic lung.

Authorities differ in opinion as to why only silica causes the formation of nodules. Most workers appear to favor the view that silicic acid, formed by solution of silica in the tissue fluid, is responsible for the inflammation and nodule formation. Others, including ourselves, incline to the view that silica itself is the causative agent. The present study was undertaken to examine the validity of these hypotheses.

Materials

1. Powders.—Quartz powders of various ranges of particle size were kindly supplied to us by Dr. C. M. Jephcott of the Ontario Department of Health in Toronto. Carbon and carborundum particles were prepared in our laboratory by fractional sedimentation of finely divided absorbent carbon (Norite A, Fisher Scientific) and carborundum dust (Fisher Scientific) by the method

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described by King *et al.* (4). The specific area and size distribution of the dusts are shown in Table I.

TABLE I

Specific area and size distribution of dusts

Type of dust	Specific gravity	Surface area of dust, sq. m/g	Proportion of particles in per cent within specified range of diameters in μ				
			<1	1–2	2–5	5–10	10–20
Quartz	2.66	1.32	10	88	2	—	—
Carborundum	3.21	0.83	31	61	8	—	—
Carbon	1.68	0.36	26	49	12	10	3

2. Silicic acid sols.—A quantity of metasilicate ($Na_2SiO_3.9H_2O$) was dissolved in saline (NaCl) and acidified to pH 6 with hydrochloric acid. The solution was diluted with saline to give a final concentration of 1 mg silica/ml in 0.9% saline and the hydrogen ion concentration was adjusted to pH 7.4 with sodium hydroxide.

3. Trypsin.—Trypsin, in crystalline form, was obtained from Armour Laboratories.

Analytical Methods

Glucose was estimated by Nelson's (5) modification of the method described by Somogyi (6).

Lactic acid was determined by the method of Barker and Summerson (7). The method of Folin and Wu (8) was used for the deproteinization of the samples. α-Amino nitrogen was estimated by the procedure described by Moore and Stein (9) with picric acid as the protein precipitant as recommended by Fleischer (10). The micro-Kjeldahl method (11) was used for the estimation of the total nitrogen.

Estimation of Antiproteolytic Activity

The antiprotease activity of the serum was measured by means of an adaptation of the methods described by Meyers *et al.* (12) and Sale *et al.* (13). A standard curve for trypsin activity was prepared by a modification of the method described by Kunitz (14) with casein as the substrate and $M/20$ potassium phosphate, pH 7.8, in 0.9% saline as the buffer. The enzyme solution was prepared by dissolving 10 mg of trypsin in 10 ml of 0.002 N HCl. A 1:150 dilution of the solution was used in the determination of the antitryptic activity of the serum. The 'trypsin unit' may be defined, for our purpose, as the concentration of trypsin or the degree of tryptic activity that liberates 10 μg of trichloracetic-acid-soluble tyrosine in 30 minutes under the conditions of the reaction.

Administration of Dusts and Taking of Samples

A single injection of an arbitrary amount (about 30 mg) of the dust (quartz, carbon, or carborundum), suspended in normal saline, was made into the peri-

toneal cavity of male albino rats of 210–250 g body weight. The particles tended to become collected into aggregates at numerous spots on the peritoneum and gave rise to an accumulation of fluid in the cavity.

In a second series of experiments, repeated injections of a saline suspension of the dusts were given intraperitoneally. A concentration of silica, much in excess of that which might be taken in by inhalation by mine workers, was used in order to produce a more intense inflammatory reaction over the surface of the peritoneum. The following quantities of the three types of dust were calculated to have a comparable total surface area: 30 mg of quartz, 100 mg of carbon, and 50 mg of carborundum. These quantities of the materials, each suspended in 1 ml of saline, were sterilized and injected, along with a small quantity of penicillin, into the peritoneal cavity of the rats. The injections were repeated on the 12th, 24th, 48th, 72nd, and 96th hour. Samples of the peritoneal exudate were withdrawn at the end of the 24th, 72nd, and 120th hour. The animals that received the carbon were given an additional injection at the end of the 120th and 144th hour, and an additional sample of the exudate was withdrawn from each animal at the end of the 168th hour. Along with the collection of the specimens of exudate, blood samples also were taken by excising a small portion of the tail. The samples of exudate and blood were permitted to clot, and the clot was rimmed and permitted to retract at room temperature. The specimens were then centrifuged and the supernatants removed for analysis.

Results

Changes in Composition of the Peritoneal Exudate and Blood Serum

The changes in the composition of the peritoneal exudate and of the blood serum with reference to the content of glucose, lactic acid, and α-amino nitrogen, upon repeated injection of quartz or carbon suspensions, are indicated in

TABLE II

Change in glucose concentration in peritoneal exudate and in serum following repeated injection of an irritant dust

Type of dust	No. animals per group	Mean glucose concentration (mg/ml) in:					
		Exudate			Serum		
		24 hr*	72 hr	120 hr	24 hr	72 hr	120 hr
Quartz	6	1.17	1.01	0.93	1.32	1.29	1.36
Carbon	6	1.23	1.33	1.19	1.40	1.49	1.41

NOTE: Necessary difference between means for significance ($P = 0.01$), 0.186.
*Hours after initial injection.

Tables II, III, and IV. It is evident from Table II that the glucose concentration in the blood serum at the 120th hour was not significantly different in the two groups whereas that in the exudate was significantly lower in the quartz-treated group than that of the carbon-treated animals.

TABLE III

Change in lactate concentration in peritoneal exudate and in serum following repeated injection of an irritant dust

Type of dust	No. animals per group	Mean lactate concentration (mg/ml) in: Exudate 24 hr*	Exudate 72 hr	Exudate 120 hr	Serum 24 hr	Serum 72 hr	Serum 120 hr
Quartz	6	0.22	0.28	0.45	0.28	0.29	0.38
Carbon	6	0.23	0.32	0.33	0.29	0.31	0.34

NOTE: Necessary difference between means for significance ($P = 0.01$), 0.089.
*Hours after initial injection.

A corresponding contrast is evident in Table III between the behavior of the lactic acid concentration in the silica and the carbon groups. Thus the level of the lactic acid at the 120th hour in the exudate of the animals that had received quartz was increased significantly compared with that of the carbon group. Despite this increase the pH of the exudate remained at about the normal level (pH 7.4–7.7). In contrast to the changes in the lactate content of the exudates, there was no significant change in the concentration of lactate in the blood serum in the two groups.

TABLE IV

Change in ratio α-amino N to total N in peritoneal exudate and serum following repeated injection of an irritant dust

Type of dust	No. animals per group	Ratio α-amino N/ total N $\times 10^3$ Exudate 24 hr*	Exudate 72 hr	Exudate 120 hr	Serum 24 hr	Serum 72 hr	Serum 120 hr
Quartz	6	4.02	4.83	12.20	4.22	4.32	4.45
Carbon	6	3.95	4.70	4.45	3.85	3.96	4.15
Carborundum	6	4.30	4.45	4.48	4.65	4.31	4.24

NOTE: Necessary difference between means for significance ($P = 0.01$), 2.94.
*Hours after initial injection.

Table IV gives the analytical values for the α-amino nitrogen in the serum and in the peritoneal exudate from three groups of animals that had received repeated intraperitoneal injections of suspensions of quartz, carbon, and carborundum dusts respectively. In the three groups the α-amino nitrogen in the blood serum remained virtually unchanged during the period of the experiment. The quartz group was conspicuous for the large increase in the α-amino nitrogen of the peritoneal exudate between the 72nd and 120th hour. There was no significant change in the composition of the exudates from the carbon and carborundum groups.

Influence of Particulate Materials on Permeability of the Peritoneum

The observation, in the case of the silica-injected group, that the α-amino-nitrogen concentration in the peritoneal fluid was greatly increased while that

of the blood serum remained unaltered pointed to a pronounced impairment of the mechanism by which crystalloidal and colloidal materials are removed from the peritoneal fluid. Further evidence of this impairment was obtained by measuring the rate of excretion of phenol red in the urine after intraperitoneal injection of a known quantity of phenol red. The animals were given repeated injections (six in all) of the quartz or carbon suspension over a period of 96 hours with administration of the phenol red 24 hours after the final injection. The results of the experiment are indicated in Fig. 1.

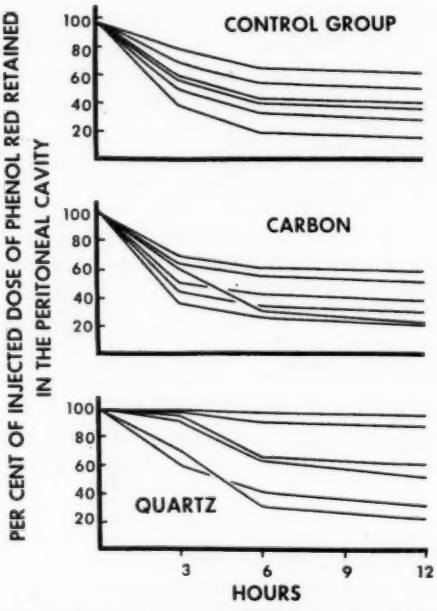

FIG. 1. Comparative retention of intraperitoneally injected phenol red in rats that had received intraperitoneal injections of saline, carbon, and quartz.

The retention of the phenol red in the case of the carbon-injected group was about the same as that in the 'control' group which received only the dye. In the individual animals, between 26 and 60% of the dye was excreted within 3 hours of the time of injection. The further excretion, by the end of the 6th and 12th hour, amounted, respectively, to about 10 and 5% of the amount administered. The quartz-injected animals, in contrast, showed a more prolonged retention of the dye.

Influence of Particulate Quartz and Carbon on the Activity of the Serum Trypsin Inhibitor

The pronounced increase in the concentration of α-amino nitrogen in the peritoneal exudate in the quartz-injected animals signifies proteolysis as an

accompaniment of the inflammatory reaction. According to the currently held view, the tryptic enzymes of the plasma and tissues (fibrinolysin, etc.) normally are held in check by 'antitryptases'. In abnormal states, when one or other of the tryptases presumably may become active, the body tends to suppress the protease by increasing the concentration of the inhibitor (antienzyme). Cliffton (15), Geust *et al.* (16), and Shulman (17) have shown that the antiprotease titer of the serum is increased in a variety of abnormal conditions including trauma, anaphylactic shock, cancer, and tuberculosis. It was of interest in our study, therefore, to measure the antiprotease activity in the serum of animals that received injections of suspensions of carbon and quartz.

The antitryptic activity of the blood serum in two groups of rats was assayed on the 1st and 5th day of the experiment to obtain a normal or 'control' value. One group then was given a daily intraperitoneal injection of a carbon suspension and the other a suspension of quartz for 5 days. The antiprotease activity was estimated on the 1st, 5th, 8th, and 14th day after the final injection was given. The results of the assays are given in Table V.

TABLE V

Changes in serum trypsin inhibitor during the intraperitoneal injection of quartz and carbon

Animal No.	Units* of trypsin inhibited					
	−5 days	0 day	1 day	5 days	8 days	14 days
	1. Group injected with quartz					
1	6.8	6.5	9.1	9.1	6.8	6.5
2	10.2	10.8	10.5	16.2	—	—†
3	9.3	9.0	9.4	10.0	10.2	10.4
4	7.4	7.2	7.0	9.8	8.1	—†
5	7.5	6.8	6.8	7.9	7.0	7.0
6	6.5	6.8	6.5	8.8	6.3	6.8
7	7.1	7.3	7.6	8.0	8.0	7.3
8	6.7	6.7	6.0	10.2	7.0	7.2
	2. Group injected with carbon					
1	5.3	5.8	6.0	6.0	6.3	6.0
2	7.8	7.3	7.3	7.1	7.6	7.5
3	7.3	8.1	7.6	7.8	7.5	7.5
4	11.2	11.5	11.8	11.6	12.0	12.3
5	9.3	9.6	9.1	8.7	8.9	8.5
6	10.6	10.5	10.7	10.2	10.8	10.3

NOTE: Incubation mixture: 2.0 ml casein solution, 0.1 ml serum (1/50), 0.6 ml phosphate–saline buffer, pH 7.4, and 0.3 ml trypsin solution, 0.067 mg/ml. Incubated for 30 minutes at 36° C.
*1 trypsin unit = amount of trypsin that will liberate 10 μg/ml of tyrosine in 30 minutes under the conditions of the reaction.
†Animals died.

The assays revealed a wide difference between individual animals with respect to the normal level of 'trypsin inhibitor' in the serum. The level in each individual, however, was found to remain fairly uniform during the control period. Comparison of the results from the two groups indicates clearly that the quartz induced a significant increase in the inhibitor activity of the serum particularly by the 5th day after the final injection, whereas carbon had little

or no effect. In the quartz-injected group, the inhibitor activity returned to the normal level by the 9th day and was found to be normal also on the 14th day.

Peritoneal Response to Silicic Acid

If the observed increase in the antiprotease activity of the serum were a response to the action of silicic acid formed by solution of the quartz particles one would expect the same response on injection of silicic acid. King and McGeorge (18) showed that powdered Rock Crystal (99.8% SiO_2), with 90% of the particles between 1 and 3 μ in diameter, was soluble in ascitic fluid to the extent of 0.5 mg per 100 ml of fluid within 24 hours at 37° C. The solubility of the quartz dust used in our study would be of a comparable order.

Silicic acid sol was prepared as described, and injected intraperitoneally, in 0.5- and 1.0-ml amounts, respectively, in two groups of six rats. The injections were given on the 1st, 12th, 24th, 48th, 72nd, and 96th hour. For 2 or 3 hours after each injection the animals appeared to be distressed but, thereafter, they resumed normal activity and food consumption. We had expected an accumulation of fluid in the peritoneal cavity but unsuccessful efforts at various times during the experimental period to obtain samples of peritoneal fluid indicated the absence of edema. At the end of the period, two animals from each group were killed by instantaneous decapitation and the peritoneal cavities examined. The viscera and peritoneum, in every case, appeared normal and there was no evidence of edema.

From this experiment it appears that silicic acid sol, even on repeated injection into the peritoneal cavity, does not give rise to the hyperemia, inflammation, and proteolysis (increase in α-amino nitrogen) that are characteristic of the response to particulate silica.

Discussion

The inflammatory response induced by silica suggests the existence of a state of hypoxia and anaerobic metabolism (glycolysis) in the inflamed mucosa. Hypoxia might account also for the impaired fluid and electrolyte exchange with accumulation of metabolites and retention of injected colloid. These responses are similar to those obtained by Menken and Warner (19) upon injection of turpentine into the pleural cavity of the dog.

Our finding, that the proteolytic response was obtained only with quartz, is in agreement with the observation of Miller and Sayers (1) that necrosis occurred only in the nodules produced in the presence of fibrogenic dusts. Among the nonfibrogenic dusts used in their studies were powdered anthracite coal and carborundum. Schepers (3) observed that the necrosis in the vicinity of silica particles was attended by the disintegration of leucocytes. These cells are known to contain proteases that become very active in slightly alkaline medium (20). Breakdown of the silica-engorged white cells at the sites of inflammation with liberation of tryptase and peptidases may account for, or contribute to, the observed proteolytic activity.

Our findings with silicic acid sol are consistent also with the observation of Banting (21) and of Gardner (22) that the intraperitoneal injection of silicic acid sol or gel does not give rise to fibrosis of the peritoneal mucosa. It would appear, therefore, that the pathogenic action of silica is attributable to the irritant action of the particles and not to that of the silicic acid formed by solution of the silica in the tissue fluid.

The principal biochemical features of the early inflammatory reaction induced by silica appear to be the activation of a protease within localized areas and a consequent decrease of the 'permeability' of the inflamed membrane at these sites. The inflammation is accompanied by the production of lactic acid. Within a few days the proteolytic activity becomes inhibited as a result of an increased production or activity of an antiprotease. It is reasonable to surmise that when the proteolysis is arrested in this manner, tissue repair proceeds by the laying down of fibrous tissue. This inference is in keeping with the histological observations of Schepers (3) and others (1, 2) that the cessation of the inflammatory phase is followed by the formation of collagen. Since native collagen is not attacked by proteases (23, 24), other than the specific collagenase, the deposition of collagen affords a permanent defence against the action of these enzymes.

Our observations may throw further light on the etiology of two common complications of silicosis. One is the almost invariable association of tuberculosis with terminal silicosis. So common is the association, especially in individuals who develop silicosis comparatively readily, that there would appear to be a common constitutional vulnerability to these diseases in certain individuals. Grob (25), several years ago, observed that the products of hydrolysis of proteins by leukoprotease (and other proteases) stimulate bacterial growth. Since the inflammatory reaction to silica is accompanied by proteolysis it is reasonable to suppose that growth of the tubercle baccilli would be favored particularly when necrosis begins in the silicotic nodules.

Another association is seen in the high incidence of silicosis among industrial workers who prepare abrasive soaps of the type that contains sodium carbonate and finely divided silica (26, 27, 28). Continuous inhalation of the soda dust would give rise to an alkaline reaction in the lung and thus intensify the inflammatory action of the silica and the proteolytic breakdown of the inflamed tissue.

We conclude from our findings and those of other workers that finely divided silica, because of its peculiar surface properties, induces in the tissues an intense inflammatory reaction accompanied by proteolysis. Unlike carbon and most other dusts, silica particles are not readily eliminated by phagocytosis and thus they tend to become aggregated into clumps. When the production of anti-enzyme causes the proteolytic phase to cease and fibrotic repair begins, the tissue tends to isolate the residual silica by surrounding the particles with collagen to form nodules.

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THE EFFECT OF HYPOPHYSECTOMY IN RETARDING THE AGEING PROCESS OF THE LOOSE CONNECTIVE TISSUE OF THE RAT[1]

MICHAEL C. HALL

Abstract

Hypophysectomy and subsequent growth hormone administration have been reported to have an effect on mucopolysaccharide metabolism. A technique is described by which comparative measurements are made of the ability of subcutaneous connective tissue to retain saline injected into it. This technique has been used in the examination of a group of hypophysectomized rats. Their tissue was found in this regard to have the character of that expected of normal rats about 100 days younger than those examined. This was the interval between the hypophysectomy and the examination of the tissues, and it is therefore suggested that hypophysectomy has an effect, specific or non-specific, that retards the ageing process of common connective tissue.

Introduction

A method of forming gelatinous bullae in the connective tissue of the pelt of the rat and the use of these bullae in making comparative estimations of the character of the connective tissue has been described (1, 2). Initial experiments in which the isolated bullae were compressed suggested that although it was not the formed fibers themselves that inhibited the passage of water through the bulla, nevertheless the histologically more fibrous tissue was better able to hold on to its injected water than was the less dense tissue. A second group of experiments examined the relationship between the rate of loss of injectant from an isolated, but uncompressed, bulla, and the weight of the animal in which it was formed. The velocity constant of fluid loss was found to bear a linear relationship to the weight of the rat.

Ellis *et al.* (3) and Dorfman and Schiller (4) have examined the effect of the growth hormone on mucopolysaccharide metabolism. It was found that S^{35} metabolism fell after hypophysectomy and rose again after growth hormone administration. C^{14} studies have shown both chondroitin sulphuric acid and hyaluronic acid levels fall after hypophysectomy, but when growth hormone is subsequently given only the chondroitin sulphuric acid level rises. If the basic premise on which the explanation of these experiments rests is correct, namely the rate of water flow from the bulla is dependent on the physicochemical organization of the connective tissue, then the changes in the metabolism of the mucopolysaccharides that have been described should be represented in changes in this rate of flow. The effect of hypophysectomy on the loose connective tissue was therefore examined.

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Contribution from the Department of Anatomy, University of Toronto, Toronto, Ontario.

Method

Young male rats were purchased already hypophysectomized from Hormone Assay Labs., Chicago. They were matched with a group of animals of the same weight from our own cages.

The examinations were performed 100 days after the hypophysectomy. The bullae were formed as previously described (2).

Histological sections were made through the pelt of the experimental and control animals. The scalp was chosen for this examination since it was unaffected by the operative procedure.

Results

In a previous experiment a plot of velocity constant against weight was made for 79 animals examined. By the method of least squares a mean was established, and this mean is used as a basis for comparison in this experiment. If the mean velocity constant of the hypophysectomized group is compared with that for animals of the mean weight of the control group it is seen that there is a considerably reduced resistance to fluid outflow from the bulla (Fig. 1). If,

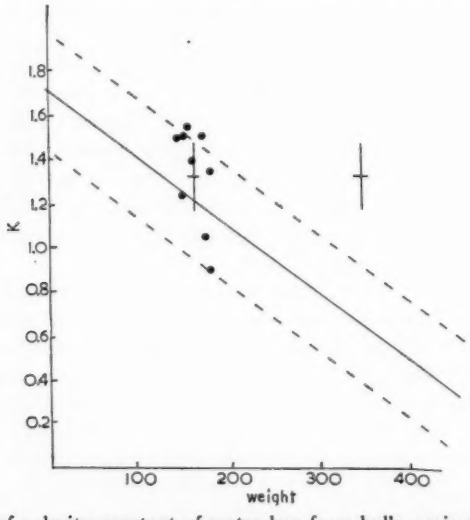

FIG. 1. Plot of velocity constant of water loss from bulla against weight of rat in grams. The velocity constants of the individual rats are shown, and the mean velocity constant is given at the left at the mean weight of the hypophysectomized group, and at the right at the mean weight of the control group. The vertical lines indicate two standard errors from the mean, and the plot is drawn against the background of the previously established K/weight relationship (the solid line is the mean K and the broken line indicates two S.E.M.).

however, the individual members of the hypophysectomized group are plotted at their own weights there is no significant deviation from the normal mean velocity constant. On the graph the mean K/weight relationship and the mean

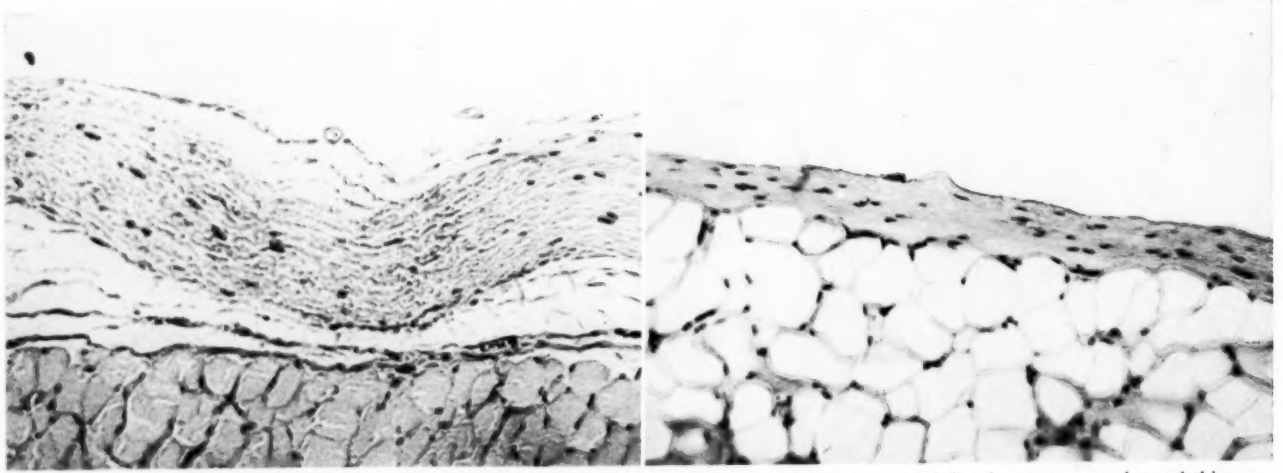

FIG. 2. Subcutaneous tissue, enlarged 200 times. The collagenic fiber bundles seen at the upper part of the picture are scantier and thinner in the hypophysectomized tissue on the right. The well-marked fat layer of the hypophysectomized animal is seen beneath the collagenic bundles.

K for the hypophysectomized group is indicated. The range of two standard errors from the mean is shown. When plotted against the control mean weight these areas are well separated, but when plotted against their own weights the overlap is very considerable.

TABLE I

Weights of hypophysectomized animals, and group maintained for purpose of control weight

Initial weights (g)		Final weights (g)	
Hypophysectomized	Controls	Hypophysectomized	Controls
90	90	145	320
95	92	150	340
97	95	150	355
100	100	155	365
100	105	160	370
105		170	
110		175	
112		180	
115		180	
Mean weight 103	96	163	350
Net change		+58.2%	+268%
Relative failure to gain weight		209.8%	

The examination of the pelt showed two striking features; there was a total reduction in thickness of about 50% and an extremely obvious fat layer occupying between a third and a half of the total pelt thickness. Microscopic examination of the dermis showed the bundles of fibers to be much thinner, and a greater cellularity of the tissue was found in comparison with the control rats. The fibers of the hypodermis also showed this thinner, finer appearance in the collagenic bundles of fibers (Fig. 2).

TABLE II

Details of rates of fluid loss from bullae

Weight (g)	% water lost at intervals of (min):					Velocity constant K
	2	4	6	8	10	
145	5.7	11.1	15.4	18.7	22.8	1.50
150	0.9	3.8	6.4	8.8	11.0	1.25
150	5.2	8.3	11.8	14.3	16.6	1.50
155	3.6	7.4	9.7	11.5	13.3	1.55
160	0.8	4.0	6.7	9.3	11.6	1.40
170	2.4	6.7	9.3	11.8	14.5	1.50
175	4.0	6.4	8.6	10.5	12.0	1.05
180	0.9	2.7	4.3	5.2	8.4	0.90
180	2.3	5.7	7.8	11.3	13.3	1.35

NOTE: Mean K, 1.33; standard error, ± 0.07; control K at group mean weight, 1.22; control K at control weight, 0.66.

Discussion

Since the anterior pituitary gland exerts a controlling effect on the other members of the endocrine system, its ablation is also reflected in changes in these other glands. In fact the success of the operation may be judged by the testicular atrophy of the subjects. Thus the diminished activity of the other glands must be borne in mind in considering the effects of the hypophysectomy, as well as the absence of the intrinsic hormones of the hypophysis. The effects of increase in anterior pituitary action are described by Iverson (5) and Asboe-Hansen (6). Practically all tissues are involved in a process characterized by hypertrophy and hyperplasia. The dermal connective tissue becomes firm and fibrous and there is a strong tendency to the formation of keloids and fibromata. The ground substance shows intense metachromasia; the collagen bundles are thick and coarse. The arteries show connective tissue hyperplasia, the bones and cartilage are thickened. There has recently been a considerable interest in hypophysectomy in the control of the growth of metastatic cancer, discussed by Hadfield (7). The histological appearance of decreased pituitary activity is discussed by Müller (8), who examined spreads of areolar connective tissue. The fibers were found to be loose-knit and smaller than normal. The ground substance was profuse, and the whole tissue cellular, which is the same appearance as reported here. Although Weinstein (9) had shown that there is a diminished rate of spread in the Duran-Reynals' reaction when performed in animals treated with anterior pituitary extract, spreading studies in the hypophysectomized animal do not seem to have found much favor. A single reference only is known and that is in regard to a single animal by Müller (8), who found no change from the normal spread. In these experiments described, the effect of the tissue of the bulla on the flow of water from it is found to be less than it would have been had they not been hypophysectomized. It is approximately the same as would be expected of normal rats of their weight, and examination of the graph shows that it is even more exactly the rate of flow expected of rats of the initial weight of the operated animals, when they were hypophysectomized 100 days prior to the examination. Thus the metabolism of the ground substance seems to have been interfered with in such a way that its normal maturation with age has been arrested. As is well known this impression of arrested age is also given by the gross appearance of the animal, which is not only smaller in over-all size, but has plumper skin and finer silkier hair than would be expected at its chronological age. Histologically this failure of maturation of the collagenic bundles, from the thin fine appearance of the young to the thick dense appearance of the adult, is a further suggestion of a retardation of the ageing process. The correlation between age and an increasing density due to collagen content in the dermis has been established by Sobel (10). McCay (11) found that rats maintained on a calorically inadequate diet, for $2\frac{1}{2}$ to 3 years, would after this time if put on an adequate diet outlive controls by about 200 days. He suggested that there was no ageing while the growth potential existed. Whether the failure of the maturation of the con-

nective tissue of the pelt is due to a specific interference in mucopolysaccharide metabolism as a result of the hypophysectomy, or is a secondary phenomenon due to a general interference with the animals' metabolism, the ablation of the gland appears to have resulted in a retardation of the ageing process.

Acknowledgments

It is a pleasure to express my appreciation to Professor J. W. A. Duckworth, in whose Department this work has been carried out, and to the Canadian Arthritis and Rheumatism Society and the J. P. Bickell Foundation, who have supported it.

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CHANGES IN LOOSE CONNECTIVE TISSUE OF THE MALE RAT FOLLOWING CASTRATION[1]

MICHAEL C. HALL

Abstract

The rate of loss of fluid from gelatinous bullae made in male rats 106 days postcastration has been compared with the rate in control rats. It has been shown to be diminished. Although the animals did not gain weight as rapidly as the controls, the hypodermis of their pelts was physically and histologically more dense.

Introduction

Methods of using gelatinous bullae in examining the physical character of common connective tissue have been described (1, 2). In a previous experiment (1) it was found that the rate of flow from a compressed bulla that had been made in the tissue of a castrated male rat was slower than that of controls. This was the only biological method found of reducing the rate of flow below the normal. This experiment has been repeated using a modified method.

Method

Seven young male rats of mean weight 104 g were castrated trans-scrotally. A sham operation was performed on a group of controls. The examination of the tissues was performed 106 days later. Gelatinous bullae were formed and examined as described elsewhere (2). Histological examination was made of the scalp; this site was chosen in order that the tissue would be unaffected by the injections.

Results

There was a relative failure of weight gain of 70% in the castrated group. The flow of fluid through the bulla was retarded, as in the previous experiment. If, on the previously established K/weight relationship, the mean velocity for the group is plotted at the control animals' mean weight, there is some overlap found between the ranges of two standard errors of both groups. If the mean K of the castrated animals is plotted at the group's actual mean weight, the difference is exaggerated considerably (Fig. 1). Histological examination showed a reduction in total thickness of the skin and an increase in subcutaneous fat. The collagenic bundles of the subcutaneous tissue in which the bullae were made were thicker and more densely packed than the controls' (Fig. 2). Significant changes in the dermis were not so apparent. The collagenic bundles did not appear to be thicker than normal and in some cases were actually finer.

[1]Manuscript received April 4, 1961.
Contribution from the Department of Anatomy, University of Toronto, Toronto, Ontario.

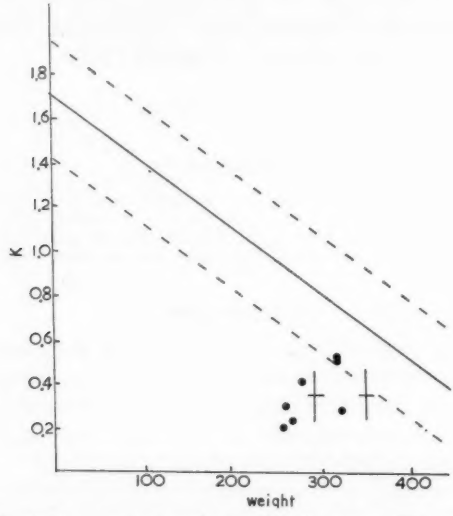

FIG. 1. Plot of velocity constant against weight in grams. The velocity constants of the individual rats are shown, and the mean velocity constant is given at the left at the mean weight of the castrated group, and at the right at the mean weight of the control group. The vertical lines indicate two standard errors from the mean, and the plot is given against the background of the previously established K/weight relationship (the solid line is the mean K and the broken lines indicate two S.E.M.).

TABLE I

Weights of castrated and control male rats

Initial weights (g)		Final weights (g)	
Castrates	Controls	Castrates	Controls
90	93	260	335
90	107	260	347
100	108	270	350
103	111	280	352
107	113	320	366
115		320	
124		325	
Mean weight 104	106	291	350
Net change		+180%	+250%
Relative failure of weight gain		70%	

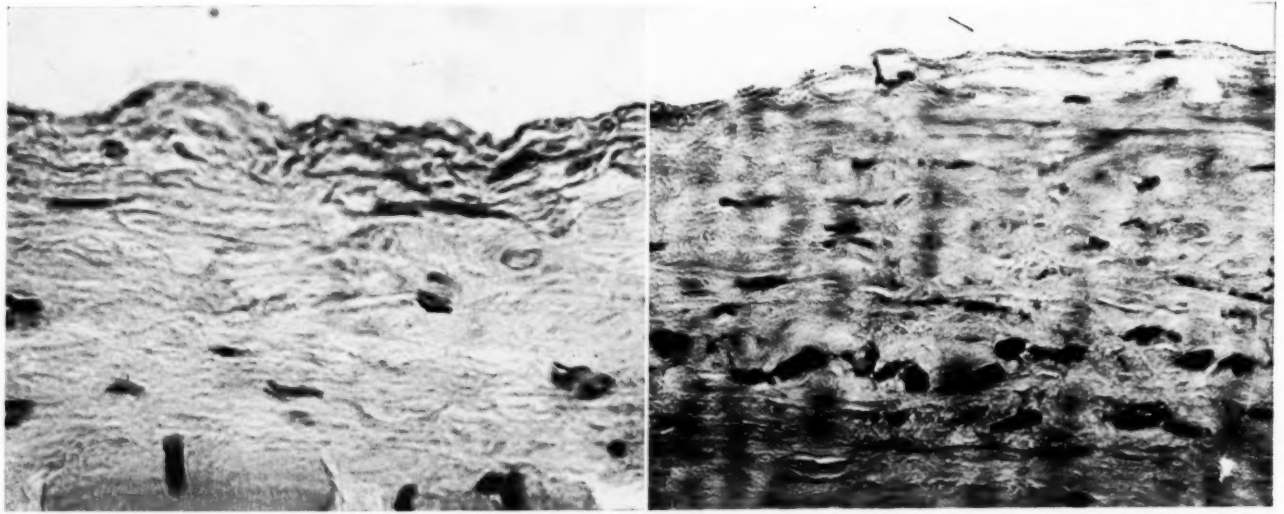

FIG. 2. Subcutaneous tissue, enlarged 350 times, showing collagenic fiber bundles. The control tissue is shown at the left and the castrate tissue at the right; the latter shows thicker more densely organized bundles.

TABLE II

Details of rates of fluid loss from bullae

Weight (g)	% water lost at intervals of (min): 2	4	6	8	10	K
260	0.7	1.5	1.7	2.3	3.0	0.29
260	0.8	1.3	1.7	1.9	2.5	0.20
270	0.6	1.0	1.6	1.9	2.4	0.24
280	0.9	1.9	2.4	3.3	4.0	0.40
320	0.7	1.6	2.8	3.4	4.7	0.52
320	1.0	1.9	3.3	4.0	4.9	0.50
325	0.6	1.3	1.7	2.4	2.6	0.28

NOTE: Mean K, 0.35; standard error, ± 0.05; control K at group mean weight, 0.84; control K at control weight, 0.66.

Discussion

These results confirm those previously obtained by the compression method. Additional evidence of the significance of the change lies in the weights of the animals. Despite the failure of normal weight gain, the tissue has matured beyond the point expected at its age, if relative abundance of fiber and ground substance is used as the criterion. Although the human following the physiological castration of the climacteric often appears to age rapidly, it is not possible from this study to conclude that the same has happened in these rats. Müller (3) found that the changes in the hypodermis, in which these bullae were made, were opposite in effect to those in the dermis. In the latter, a loss of density of the tissue was reported. This appearance was sought in the tissues examined from the castrates, and was not found as obvious in these rats as Müller described. However, she apparently was describing changes in the female which may well be different from those in the male.

Acknowledgments

It is a pleasure to express my appreciation to Professor J. W. A. Duckworth, in whose Department this work has been carried out, and to the Canadian Arthritis and Rheumatism Society and the J. P. Bickell Foundation, who have supported it.

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THE VARIATION WITH BODY WEIGHT OF THE WATER-BINDING PROPERTIES OF COMMON CONNECTIVE TISSUE[1]

MICHAEL C. HALL

Abstract

A standard volume of saline was introduced at standard pressure into the reflected subcutaneous tissues in 79 male albino rats, forming gelatinous bullae. The rate at which the saline was lost from the bullae after it had been dissected free was estimated by periodic weighings, and a velocity constant of this rate was derived. There was found to be a linear relationship between this constant and the weight of the animal. It is believed that the injected saline combines with the connective tissue, and that the alteration with body weight of the rate of loss of the saline subsequent to the injection is an expression of the change of the water-binding ability of this tissue.

Introduction

The use of gelatinous bullae to examine the physicochemical character of connective tissue has been described (1), and reasons have been given for the belief that the varying ability of the bullae to retain the newly introduced water is related to the water-binding properties of this tissue. A further experiment has been performed, in a modified manner, to examine more exactly the relationship between body weight and the rate of liberation of water from the bullae. In the previously described experiment bullae were made with 3 to 5 ml of saline and compressed in a standard fashion; a great disparity was found in the ability of tissues of animals of widely separate weights to retain the injected saline. Bullae of smaller size were made in this experiment allowing easier comparison between the two extremes of tissue examined. They were allowed to lose water without being compressed.

Method

Seventy-nine male albino rats were fed on standard diet. The animals were lightly anaesthetized with intraperitoneal Nembutal, introduced low in the midline of the abdominal wall in order not to affect tissue that would be examined. The level of anaesthesia was not lowered below that required for a pain-free incision, with the intention of affecting the blood supply to the area under examination to the minimum possible extent. The pelt was lifted up on the ventral surface of the animal and opened along the belly slightly to the left of the midline. The pelt on the right side was then stripped away by blunt dissection. By prolonging the incision to the right at the upper and lower extents of the abdomen, a flap of pelt was made whose blood supply was intact. This was then lightly pinned to the plastic-covered wooden operating table with the subcutaneous surface uppermost. The instrument used to ensure steady injection of solution was the "Syringe Driver" adaptation of the Phipps and

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Contribution from the Department of Anatomy, University of Toronto, Toronto, Ontario.

Bird kymograph. A 5-ml syringe was used at the driver's speed No. 4, delivering 0.58 ml per minute. The syringe was connected through a 5-in. strip of thick-walled rubber tubing to a gauge 25 needle with short bevel and 1-in. shank. Using binocular magnifying "Dualoupes", the needle was inserted into the center of the subcutaneous tissue with minimal trauma. The syringe driver was turned on immediately prior to the insertion of the needle to ensure that no air was introduced. The solution was normal saline in every case, at pH 7, checked with a Beckman pH meter. A bulla (Fig. 1) of 1.0 ml was made. This bulla was excised very gently from the surrounding tissue and transfixed with a fine sewing needle, which was embedded in a small block of light cork. Threaded through this cork was a loop of fine wire permitting the cork, bulla, and needle to be suspended from the middle of the loop of the weighing pan on a "Sartorius Semi-Micro" balance. Water is exuded continuously from the bulla and gradually forms a drop which falls from it. In order to eliminate as much as possible the error due to the accumulation of water prior to the falling of the drop, the bulla was gently touched with the edge of a piece of filter paper at its lower pole only, in a standard fashion, exactly ½ minute prior to taking a reading. The weight readings were taken every 2 minutes for 10 minutes, i.e. at 0, 2, 4, 6, 8, 10 minutes. The bulla was then removed from the needle, placed on a weighed fragment of filter paper, and left in a vacuum desiccator for 24 hours. The weight of the filter paper subtracted from the weight of the filter paper and tissue gives the weight of the dried tissue which composed the solid portion of the bulla. From this is derived the weight of water contained by the bulla at the various times of weighing. The weight of water remaining in the bulla at time t was then expressed as a percentage of the weight of the water in the bulla at time 0. A plot was made of the percentage of water remaining against time in minutes. The atmospheric temperature and humidity were not controlled. The plot that has been used in this experiment is, in fact, the initial velocity of the saline loss from the bulla. In the heavier animals there was no difficulty in drawing a line to fit all points, including zero. In the lighter animals, however, difficulty was experienced in some cases and the line was then drawn that would best fit the maximum number of points in the later minutes of the experiment. This method was chosen since it is believed that some of the water lost rapidly from the connective tissue of the lightest animals had in fact never become "bound water", for the tissue in such animals already contained a high percentage of water. This belief is further supported by the apparent biphasic appearance of many of these graphs (Fig. 2) in which, during the first few minutes, a curve is seen that expresses the rate of loss, but the rate of loss in the final few minutes is linear. A velocity constant of rate of water loss, K, was derived from the slope of the line.

Histological sections were made of the tissue removed from each animal as follows:

(*a*) Skin from scalp. (This site was chosen so that the skin would not have been affected by the injection.)

PLATE I

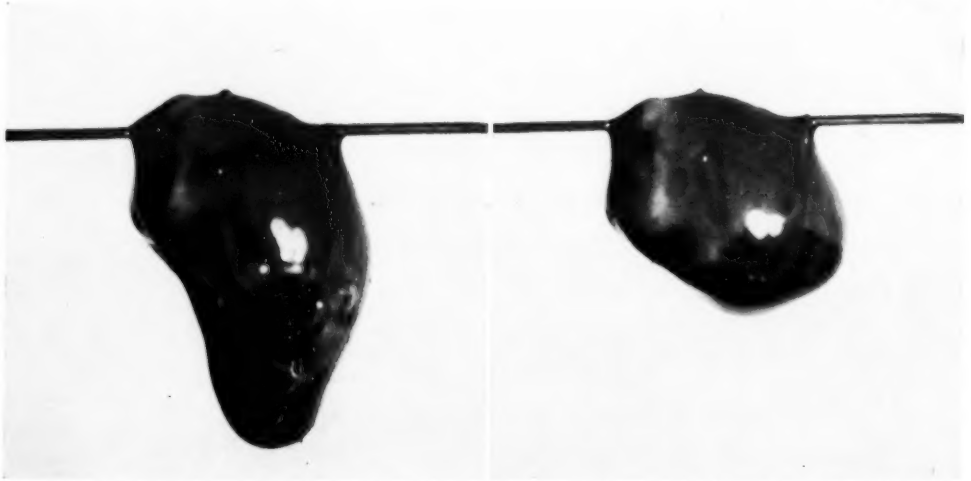

FIG. 1. (Left) Isolated bulla (colored for photographic purposes) suspended from a wire. (Right) Same bulla with lower half cut away to demonstrate continued retention of water by the remaining tissue.

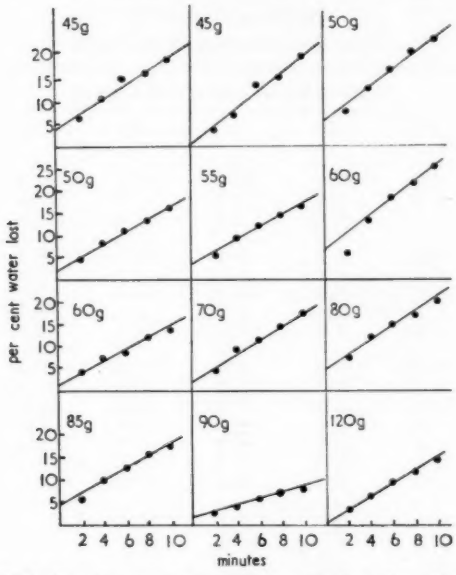

FIG. 2. Individual plots of percentage water lost against time, in lighter animals.

(*b*) Rectus abdominis muscles, upper, to show perimuscular fascia and perivascular fascia of superior epigastric artery.

(*c*) Frozen sections of bullae made in opposite side of ventral pelt after completion of weight studies.

Results

The percentage loss of water at 2-minute intervals, with the calculated velocity constant, is given in Table I. These velocity constants were plotted against the weights of the animals (Fig. 3). By the method of least squares, a

TABLE I

Water loss from experimental bullae

Weight (g)	% water lost at intervals of (min):					
	2	4	6	8	10	*K*
45	6.5	12.0	15.5	16.8	19.8	1.60
45	3.9	7.3	14.0	15.9	20.8	1.80
50	8.0	13.6	17.8	21.3	24.3	1.70
50	4.5	8.0	11.0	13.5	16.3	1.40
55	5.5	9.5	12.2	14.8	16.8	1.40
55	5.4	8.0	12.4	14.5	17.3	1.50
60	6.0	13.5	18.9	22.2	25.9	1.40
60	4.0	7.1	8.8	12.0	13.8	1.40
70	4.5	9.2	11.7	14.7	17.7	1.40

TABLE I (*Continued*)

Water loss from experimental bullae

Weight (g)	% water loss at intervals of (min.): 2	4	6	8	10	*K*
80	7.3	12.0	15.0	17.7	20.5	1.60
85	5.5	9.9	12.9	15.7	17.7	1.40
90	2.7	4.1	6.0	7.0	8.0	1.20
120	3.0	6.4	9.6	12.0	14.5	1.50
120	3.2	6.4	8.9	11.7	15.0	1.30
125	4.7	8.0	10.3	13.5	15.7	1.30
125	1.3	3.4	6.8	10.3	12.5	1.50
125	5.5	9.8	12.8	15.4	18.0	1.40
130	2.5	7.5	8.9	12.0	14.5	1.40
130	1.0	3.0	5.7	8.4	11.5	1.30
130	3.8	6.0	7.5	9.5	11.4	1.30
140	2.0	4.4	7.0	10.0	12.0	1.30
140	2.0	3.7	6.4	8.8	11.3	1.20
145	4.6	8.7	12.5	14.9	18.2	1.50
145	3.7	6.5	9.0	11.0	12.7	1.40
155	3.7	6.1	8.3	10.4	12.7	1.40
155	4.8	8.7	12.0	14.3	17.0	1.40
160	6.2	9.0	11.9	13.4	15.6	1.10
162	7.0	8.0	10.5	13.0	14.5	1.12
172	12.0	16.0	20.2	23.5	25.0	1.10
178	4.0	6.2	8.0	10.5	12.0	1.20
180	6.5	8.0	11.2	12.5	16.0	1.04
195	3.0	6.5	8.2	11.0	12.5	1.04
197	6.5	8.0	11.0	14.2	16.0	1.40
200	17.0	19.0	21.5	23.5	26.0	1.08
230	1.9	3.7	6.3	11.2	13.9	1.10
230	2.5	5.2	7.5	9.2	11.2	1.00
245	0.7	1.8	3.3	4.5	5.3	0.70
250	3.5	6.0	8.2	10.3	12.0	1.10
262	3.0	5.0	6.5	7.5	10.2	0.94
265	1.6	4.2	6.0	7.5	9.8	1.00
265	1.8	3.0	5.2	7.0	9.0	0.90
270	7.0	9.2	11.5	13.0	15.5	0.96
270	3.0	5.7	6.5	11.0	13.5	1.04
275	5.5	7.0	9.2	10.5	12.0	0.80
290	8.0	10.0	12.2	14.0	15.8	0.96
296	2.7	5.0	7.5	9.2	12.0	0.94
296	7.5	8.5	10.0	11.5	15.7	0.72
298	2.7	3.5	4.2	5.0	6.2	0.66
305	1.5	2.5	4.7	5.0	6.5	0.64
324	2.0	3.2	4.7	6.5	8.0	0.80
324	6.5	8.0	10.2	11.5	13.0	0.80
325	3.2	4.0	4.7	5.7	7.0	0.48
335	6.5	8.2	11.0	12.0	14.7	0.84
338	2.7	3.7	5.5	7.0	8.2	0.80
340	1.7	3.0	4.0	4.7	6.0	0.52

TABLE I (*Concluded*)

Water loss from experimental bullae

Weight (g)	% water lost at intervals of (min): 2	4	6	8	10	*K*
352	2.0	2.8	4.0	4.7	6.0	0.60
352	0.5	1.7	2.5	4.7	6.0	0.56
355	0.5	1.5	2.2	3.0	4.2	0.52
357	1.7	2.7	3.5	3.7	4.2	0.44
360	2.0	3.8	5.2	7.2	9.0	0.84
360	1.5	2.0	3.5	4.0	6.2	0.64
380	1.5	2.7	3.5	4.0	4.7	0.48
380	17.5	18.0	19.5	20.0	21.0	0.48
390	3.2	5.0	6.7	8.0	10.5	0.84
390	1.5	2.2	3.5	4.0	5.7	0.56
390	0.5	2.0	3.0	4.2	5.7	0.56
410	0.9	1.4	2.5	3.5	4.5	0.40
410	0.9	1.4	2.0	2.2	2.9	0.30
410	2.0	3.7	5.2	6.3	7.2	0.60
410	1.0	1.8	2.5	3.5	4.3	0.40
415	0.6	1.7	3.4	5.9	6.3	0.60
415	1.1	2.5	4.0	5.0	6.5	0.60
435	0.3	1.0	1.8	2.2	3.0	0.30
435	0.8	1.3	1.7	2.3	2.8	0.30
450	1.0	2.0	2.5	3.5	4.3	0.40
450	0.9	2.0	3.7	4.5	5.2	0.50
450	0.2	2.3	4.4	5.9	7.7	0.60
470	1.0	1.8	2.8	3.7	5.0	0.50
470	1.3	1.5	2.4	3.0	3.6	0.40

mean velocity constant was calculated whose slope was -0.00295, and the standard error from the mean of this slope was ± 0.135.

The tissues removed from these animals show a progressive change of all forms of connective tissue examined in relation to the animal's weight (Figs. 4–8). Sections of the skin in the youngest animals show small bundles of collagenic fibers isolated by a profuse ground substance. The fibers of the bundles, although they are identifiable as such, are well bound together. Following through the dermis as weight increases there is found a progressive thickening of the collagenic bundles, which leads to an increase in density of the fibrous tissue of the dermis at the expense of the ground substance. The volume of ground substance thus diminishes in balance with the increase in formed fibers. Differences in density between the already dense fibers of the dermis of the 350-g rat and those of the 450-g rat are difficult to appreciate. However, the progressive increase in density is seen more easily in the subcutaneous tissue which is both denser and thicker in the heavier animal. Frozen sections were made of the subcutaneous tissue in the bullae, and these show the same progressive increase in the appearance of the collagenic bundles, which appear in these sections as a greater number of collagenic fibers composing each

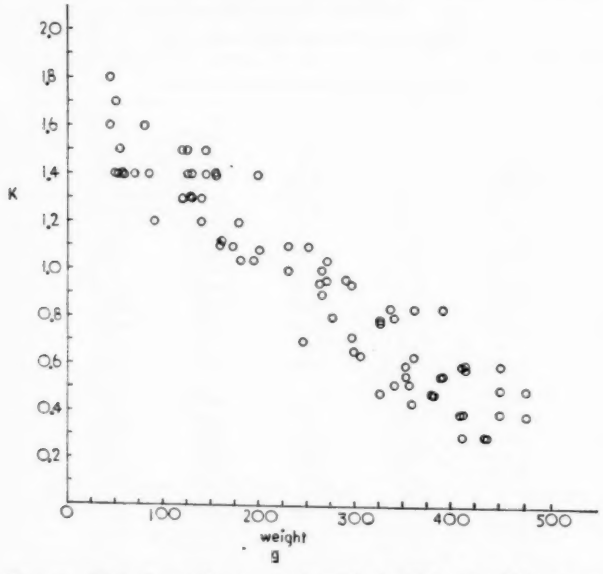

FIG. 3. Plot of velocity constants against weight of rats in grams.

bundle. It can be seen that there is also an increase, not only in the number of fibers, but in the thickness of the individual fiber, suggesting that there is an increase in the number of fibrils per fiber.

Discussion

The experiment has established a linear relationship between body weight and the ability of common connective tissue to retard the outward flow of saline injected into its substance. The experiment bears some similarity to the spreading reaction of Duran-Reynals (2) but has the significant difference that only the properties of the substance of the bulla are being examined, and there is no "barrier effect" of surrounding tissue to be taken into consideration. Fragmentation of the bulla, as described, does not result in a sudden loss of contained water, and for reasons previously stated (1) it is believed that the injected saline has become part of the aqueous phase of the colloidal system. Gortner (3) has described experiments performed on wheat leaves in which it was found that whereas sap could be expressed easily in summer, in the winter, although the actual water content had not changed, sap could only be expressed with great difficulty. From this type of experiment was derived the concept of "bound" and "free" water, and of a mutual balance between them. From the conception of tightly bound water, not available for solution of electrolytes etc., has developed the misconception of water that is quite free and that would drain from the tissues if they were opened. That this is not the case, and that there

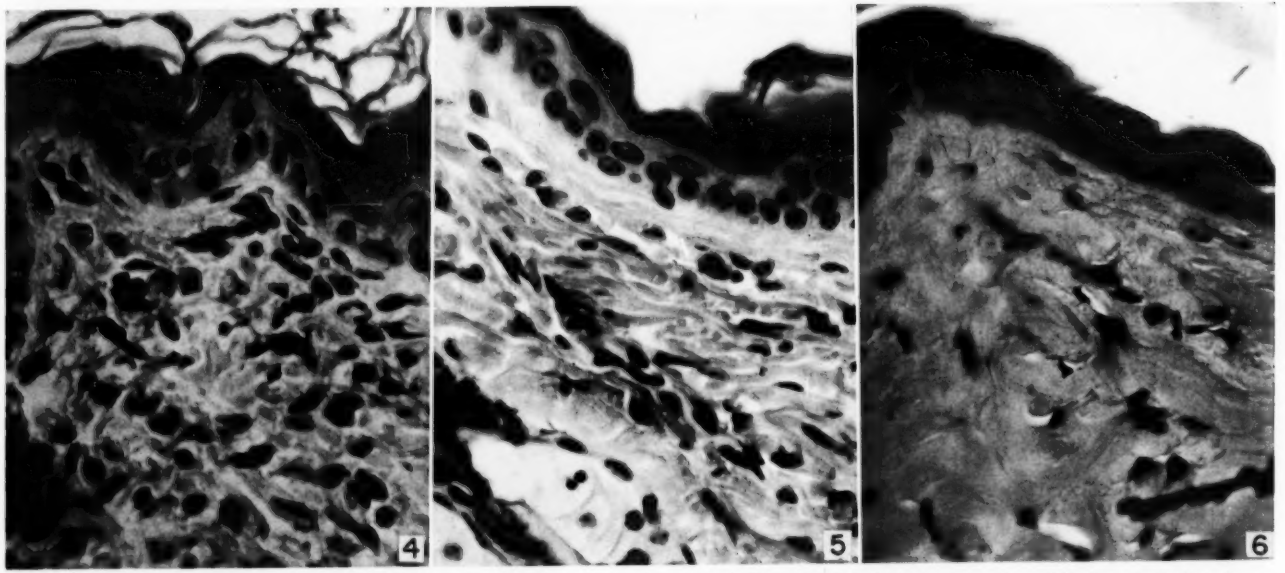

FIG. 4. Dermis of 20-g rat, enlarged 300 times. If this figure is compared with Figs. 5 and 6, it is seen that as weight increases there is a progressive increase in both thickness and numbers of collagenic bundles in the dermis.
FIG. 5. Dermis of 120-g rat. 300×
FIG. 6. Dermis of 220-g rat. 300×

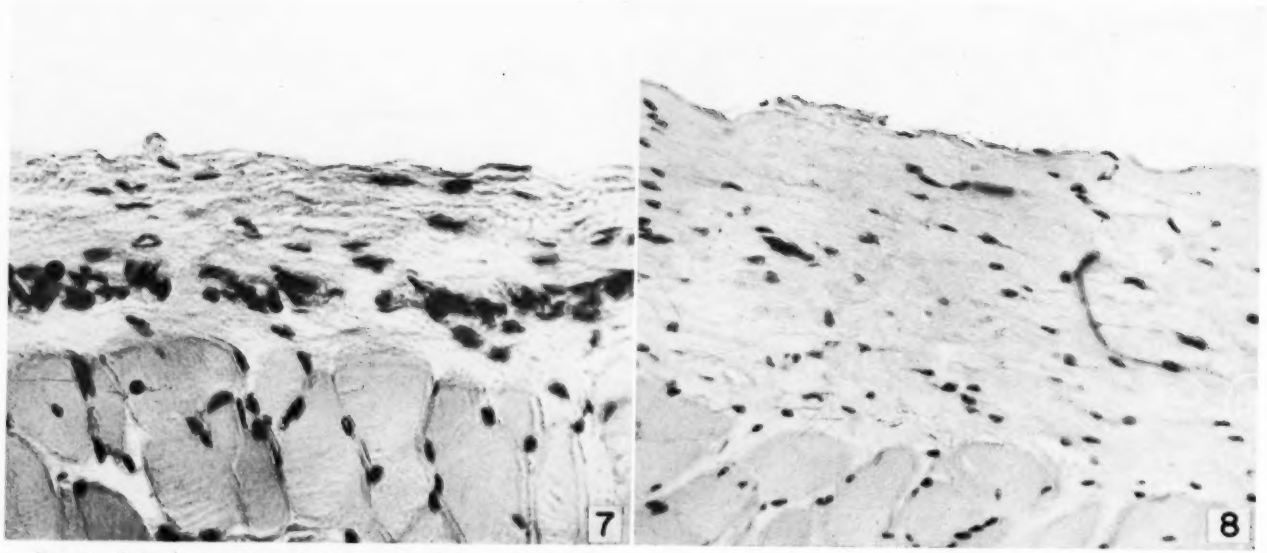

FIG. 7. Subcutaneous tissue of 350-g rat. 250×
This is arranged so that the collagenic bundle area in which the bullae were made is at the top of the photograph, and the panniculus carnosus muscle layer is seen beneath. Compared with Fig. 8 the collagenic bundles are more loosely organized.
FIG. 8. Subcutaneous tissue of 450-g rat. 250×

is in fact normally no free water at all was demonstrated by McMaster and Parsons (4), who showed that there was no diffusion of an injected dye through the dermis unless introduced with force. The water in the tissue is therefore the dispersion medium of a gel. Thus the simple division between "bound" and "free" water is inadequate, separating in fact the dispersion medium only into two groups of "available for solution of electrolytes" and "not available for solution of electrolytes". It takes no notice of the degree to which the rest of the water of the gel is trapped, and it would be of help in our understanding of the function of connective tissue if the term "free water" was abandoned and the earlier qualification "firmly bound" was used to contrast with the term "loosely bound".

The relationship of age to body weight in the albino rat is described by Donaldson (5), and from his data and that of others it is known that an increase in age is accompanied by an increase in body weight, although the rate of increase falls off rapidly as age advances. Therefore one may regard opposite extremes of the weights of the rats as representing older and younger animals, and conclude in general terms that the velocity constant is altering with increase in age.

There is a considerable volume of work supporting the histological changes that have been described. Gross and Schmidt (6) in electron microscopic studies showed that the formed fibers in the young animal are fewer and shorter than in the old, and that in the process of ageing there is a relative decrease in the ground substance and a thickening of the collagenic bundles. Similar changes are reported by Gersh and Catchpole (7), and the chief area of dispute seems to be in regard to possible atrophy of the collagen bundles in senescence. Increase in fibrous character of articular cartilage is described by Ham (8) and of the nucleus pulposus of the intervertebral disks by Sylven and associates (9). In chemical extraction studies, Sobel (10) found that collagen is deposited at an increasing rate as the animal grows, but the hexosamine increase parallels the weight gain, so that with growth the ratio between hexosamine and collagen diminishes. He observed that there was a linear relationship between the log of the "H/C" ratio and the patient's age in skin biopsies, and has proposed this as a measure of biological, as opposed to chronological, age. Schmidt (11), Hvidberg (12), and Boas and Foley (13) have also shown that the concentration of hexosamine in the dermis of the rat decreases with age, and may be correlated with the water content. Stidworthy and associates (14) showed a decrease with age of rib chondroitin sulphate, and Dyrbye (15) has shown a decrease with age of the S^{35} uptake in human arteries. Changes in the collagen fiber with age are critically reviewed by Bensusan (16). Differences in cross-bonding, shrink temperature, and swelling in acid are found.

Thus, throughout life there is a progressive alteration in the physical and chemical nature of the connective tissue, and, since the loosely bound water of connective tissue is dependent on the organization of this heterogenous colloidal system, it too progressively alters.

Acknowledgments

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THE UTILIZATION OF RIBOFLAVIN GIVEN PERIODICALLY TO THE RAT APART FROM THE DIET[1]

A. B. Morrison, E. J. Middleton, and J. A. Campbell

Abstract

Male weanling rats were depleted of riboflavin reserves and given limiting amounts of riboflavin in the diet throughout the day, or at 1-, 2-, and 4-day intervals by stomach tube. Riboflavin given once a day by stomach tube was as efficiently utilized for growth as the same amounts of the vitamin given daily in the diet. Animals given equivalent amounts of riboflavin at 2- and 4-day intervals, however, showed a stepwise reduction in weight gain, food intake, and efficiency of food utilization. The efficiency of utilization of riboflavin given every 4 days varied with the size of the dose.

In metabolic studies, rats given riboflavin every 4 days excreted more of a dose of C^{14}-labelled riboflavin in the urine and feces, and retained less of the dose in the liver and carcass, than those given the vitamin daily. Administration of riboflavin at 4-day intervals had no effect on liver and carcass riboflavin levels.

It was concluded that riboflavin given as a single daily dose is as well utilized by the rat as that consumed with the diet in several small doses over a 24-hour period. Administration of the vitamin at greater than daily intervals reduced the efficiency with which it was utilized.

Introduction

Although vitamin preparations are dispensed in an increasing variety of dosage forms to large numbers of people, relatively little is known of the utilization of vitamins given separately from the diet. Sarett and Morrison (1) concluded that limiting amounts of thiamine, riboflavin, pyridoxine, and pantothenic acid administered to rats separately from the diet each day were as well utilized as those incorporated in the diet. Earlier work by Macko *et al.* (2) indicated that guinea pigs given ascorbic acid daily grew more rapidly than those receiving the same amount of the vitamin at semiweekly or weekly intervals. In additional studies, Macko (3) found that vitamin A given once a week to rats was as well utilized as the same amount of the vitamin given in seven daily doses, but thiamine and vitamin D were better utilized if given daily rather than at weekly intervals. Recent interest in sustained–release vitamin preparations (4, 5) has prompted further studies on the utilization of vitamins given at periodic intervals. Although urinary excretion studies in man provide valuable indirect evidence on vitamin utilization, studies in the growing rat were also felt to be of interest and to provide another approach to the problem. It should of course be realized that there may be differences in the way the rat and humans utilize vitamins. In the experiments reported herein, data on weight gain, food intake, urinary and fecal excretion of riboflavin, and tissue riboflavin levels were obtained in growing rats given riboflavin at periodic intervals apart from the diet.

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Contribution from the Food and Drugs Laboratories, Department of National Health and Welfare, Ottawa, Canada.

Experimental

The basal diet contained 18% vitamin-free casein, 62.73% sucrose, 10% corn oil, 5% non-nutritive cellulose, 4% U.S.P. XIV mineral mixture, and the following vitamins per 100 g: thiamine HCl, 0.5 mg; pyridoxine HCl, 0.5 mg; calcium pantothenate, 1.5 mg; niacin, 2.0 mg; *p*-aminobenzoic acid, 4.0 mg; biotin, 0.02 mg; folic acid, 0.2 mg; *i*-inositol, 50.0 mg; choline bitartrate, 200.0 mg; menadione, 0.2 mg; vitamin B_{12}, 0.002 mg; vitamin A, 500 I.U.; calciferol, 100 I.U.; DL-alpha tocopherol, 10 I.U. In order to minimize vitamin losses, the diet was kept refrigerated. Male weanling Wistar rats of the Food and Drugs colony were housed individually in screen-bottom cages kept in an air-conditioned room maintained at 74° to 76° F, and given the basal diet for a 17-day pretest period. They were then divided into groups and given the various experimental diets. In experiments 1 and 2, 10 tetrads of comparable animals were selected on the basis of body weight. Animal A of each tetrad received, ad libitum, the basal diet supplemented with 125 μg of riboflavin per 100 g of diet. This amount is approximately one-half of that required by the growing rat (6). Animals B, C, and D continued to receive the riboflavin-free basal diet, ad libitum. Animal B of each tetrad was given, each day, the same amount of riboflavin as that consumed by animal A the previous day. Animals C and D of each tetrad received the same total amounts of riboflavin consumed by animal A, but at 2- and 4-day intervals, respectively. The vitamin was administered to animals B, C, and D in aqueous solution by stomach tube. To facilitate its administration, a solution was prepared containing, per ml, the amount of riboflavin in 10 g of the diet consumed by animal A. To equalize the effects of handling and treatment, animal A of each tetrad was given 0.5 ml of water daily by stomach tube. The animals were weighed individually at intervals during the 16-day growth phase of the experiments, and records were kept of the amount of food consumed by each rat. Comparable animals from each group were then placed in metabolism cages and urine was collected in opaque bottles at daily intervals over a 4-day period. Feces were also collected in experiment 2. The excreta collected each day were frozen until analyzed for riboflavin. At the end of the metabolic studies, the animals were killed by ether anesthesia, exsanguinated, the livers were removed and weighed, and liver riboflavin levels were determined. Riboflavin in urine, feces, and liver was determined by appropriate modifications of the U.S.P. fluorometric procedure (7).

In experiment 3, six comparable pairs of animals were selected at the end of the pretest depletion period. Both animals of each pair continued to receive the riboflavin-free basal diet, ad libitum. Animal A of each pair was given 10 μg of riboflavin daily, by stomach tube, for 8 days, whereas animal B of each pair received 40 μg of the vitamin by stomach tube on days 1 and 5. The animals were weighed individually each day, and records were kept of the amount of food eaten by each rat. On day 8, the animals were placed in metabolism cages for studies with radioactive riboflavin, labelled in the number

2 position. Animal A of each pair received 10 μg of C^{14}-labelled riboflavin on days 8, 9, 10, and 11, whereas animal B received 40 μg of the labelled vitamin on day 8 only. Urine and feces were collected daily for 4 days and frozen until analyzed. One of the A animals lost weight during the metabolic study, possibly because of aspiration pneumonia, and the data for this animal and its pair mate were discarded. At the end of the 4-day metabolic study, the remaining animals were killed by ether anesthesia, exsanguinated, and the livers removed and weighed. Liver, fecal, and urinary riboflavin levels were determined as in experiments 1 and 2. In addition, carcass riboflavin was also determined in aliquots of the ground carcasses. Total radioactivity was determined on the tissue and excreta samples, using a Tracerlab TGC-14 thin window flow detector operated in the Geiger region.

Experiment 4 was conducted to obtain a quantitative estimate of the efficiency of utilization of riboflavin given at 4-day intervals apart from the diet. From experiments 1 and 2, it was estimated that when the vitamin was given at 4-day intervals, it was utilized approximately 40% as efficiently as when given daily. To test this hypothesis animals were given the riboflavin-free basal diet for the 17-day pretest period. Thirty pairs of comparable animals were then selected, on the basis of body weight. One number of each pair was given the basal diet supplemented with graded levels of riboflavin, to provide 50, 100, or 200 μg of the vitamin per 100 g of diet. The second member of each pair continued to receive the riboflavin-free diet ad libitum, and, in addition, was given, by stomach tube at 4-day intervals, 2.5 times the amount of riboflavin eaten by its pair mate. The animals receiving the diets containing added riboflavin were given 1 ml of water, by stomach tube at 4-day intervals, to equalize the effects of handling. The animals were individually weighed at intervals during the 12-day experimental period and records were kept of the amount of food consumed by each rat. The riboflavin content of the diets containing added riboflavin was checked by fluorometric assay (7).

The data of the experiments were analyzed by appropriate statistical procedures (8).

Results and Discussion

Experiments 1 and 2

The results of experiments 1 and 2 are shown in Table I and Fig. 1. The animals which received riboflavin once daily by stomach tube grew at comparable rates to those given the vitamin in the diet, and consumed similar amounts of food. These results are in agreement with those of Sarett and Morrison (1), who gave four B vitamins in the diet, or separately from it, daily. The rat is normally a "nibbler" which eats its food in several portions during the day. Under these circumstances, the utilization of riboflavin for growth was no more efficient than when the vitamin was given once daily. In previous studies with human subjects, Chapman and Campbell (9) and Morrison *et al.* (4) observed that the total urinary excretion of riboflavin was not affected by giving the total dose in a number of divided doses over a period of several hours. The

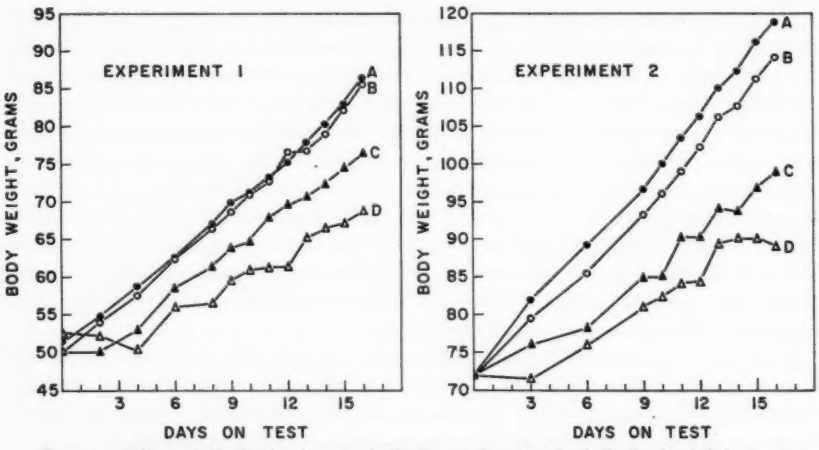

FIG. 1. Effect of riboflavin given periodically on the growth of riboflavin-deficient rats: A, in the diet; B, by stomach tube every day; C, by stomach tube every 2 days; D, by stomach tube every 4 days.

present results furnish further evidence that giving riboflavin in several small doses during the day is of no physiological advantage over giving it once a day.

The C rats, which received riboflavin every other day, grew less rapidly than those given the vitamin daily. This difference approached significance in experiment 1 and was significant in experiment 2 ($P < 0.01$). In both experiments, the animals which were given riboflavin every 4 days grew at a significantly reduced rate and consumed significantly less food than those given the vitamin daily. They also exhibited a significantly reduced efficiency of food utilization. Examination of the growth curves found in the two experiments (Fig. 1) showed that the A and B rats, which received riboflavin daily, grew at a relatively constant rate during the experiments. The animals receiving riboflavin every other day (C rats), or every 4 days (D rats), exhibited growth curves characterized by growth spurts on the day when riboflavin was given, followed by much slower growth until the next dose was given. The rhythmic response to riboflavin observed in these studies brings to mind that obtained by Waibel *et al.* (10), in chicks given thiamine by injection every 2 or 4 days.

The results of the metabolic studies carried out in experiments 1 and 2 are also summarized in Table I. The values shown for urinary and fecal excretion represent the total excretions during the 4-day period. Urinary excretion of riboflavin in both experiments did not appear to be significantly influenced by treatment, although the amount excreted in experiment 2 was less than that in experiment 1. The fecal excretion of riboflavin in experiment 2 was not significantly different in the groups given riboflavin in the various ways. Liver riboflavin levels in the first experiment were higher than in the second, but were not influenced in either study by the way in which the vitamin was administered.

TABLE I

Effects of periodic administration of riboflavin on growth and excretion of riboflavin in rats

	Experiment 1				Experiment 2			
	In diet	Once/day	Once/2 days	Once/4 days	In diet	Once/day	Once/2 days	Once/4 days
(A) Growth studies								
No. of animals	10	7	7	6	10	9	10	9
Initial weight, g	51	52	51	51	72	72	72	72
Weight gain	35±2.5*	35±2.3	26±2.0	16±4.0	47±2.4	42±2.0	27±1.4	17±3.7
Food intake, g	108	104	98	87	137	140	120	105
Food efficiency, g gain/100 g food	32.1	33.4	26.7	19.2	34.1	29.9	22.3	16.0
(B) Metabolic studies								
No. of animals	5	3	3	3	4	4	4	4
Riboflavin dose, μg	46	40	40	40	38	40	40	40
Riboflavin excretion in:								
Urine, μg	15	14	9	13	7	7	5	6
Feces, μg	—	—	—	—	10	12	10	10
Liver riboflavin, μg/g	15.3	16.7	13.3	16.0	6.2	6.9	6.8	6.3

*Standard error of the mean.

Experiment 3

The results of experiment 3 are summarized in Table II. In this experiment, as in the first two experiments, rats given riboflavin at 4-day intervals exhibited reduced weight gain, food intake, and efficiency of food utilization, as compared

TABLE II

Excretion of radioactive riboflavin by rats given the vitamin daily or at 4-day intervals

	Riboflavin administered	
	Daily	Every 4 days
(A) Growth studies		
No. of animals	5	5
Initial weight, g	58	58
Weight gain, g	22 ± 2.9*	13 ± 3.8
Food intake, g	56	43
Food efficiency, g gain/100 g food	39.3	30.2
(B) Metabolic studies		
No. of animals	4	4
Riboflavin dose, μg	40	40
Riboflavin excretion in:		
Urine, μg	5.7	5.3
Feces, μg	16.7	21.2
Tissue riboflavin in:		
Liver, μg	9.0	8.8
Carcass, μg	1.2	1.1
C^{14} excreted in:		
Urine, c.p.m./μg riboflavin	102 (83–122)†	191 (145–307)
Feces, c.p.m./μg riboflavin	66 (45–97)	167 (122–248)
Liver, c.p.m./μg riboflavin	37 (31–40)	28 (19–35)
Carcass, c.p.m./μg riboflavin	33 (28–40)	21 (18–26)

*Standard error of the mean.
†Range of values.

with those given the vitamin daily. In further agreement with experiments 1 and 2, the urinary and fecal excretions of riboflavin during the 4-day metabolic period were not markedly different in animals given daily doses of the vitamin than in those given riboflavin at 4-day intervals. Although the results shown in Table II represent the total excretions during the 4-day test period, it should be noted that the amounts of riboflavin found in the feces and urine of the animals given the vitamin at 4-day intervals were highest the day after dosing, and fell rapidly thereafter. The urinary and fecal excretions of riboflavin in the animals given the vitamin daily tended to show less day-to-day variation. Liver and carcass riboflavin levels were not significantly different in animals given the vitamin daily than in those given riboflavin at 4-day intervals.

The results obtained in experiment 3 with C^{14}-labelled riboflavin are believed to provide a more precise estimate of the metabolic fate of the administered doses than do measurements of total urinary and fecal riboflavin excretion. The animals given the vitamin at 4-day intervals showed higher urinary and fecal excretions of the test dose, and lower tissue retention of the dose, than those given riboflavin daily. These results confirm previous indications of less

efficient utilization of riboflavin given at 4-day intervals. It is possible that the higher fecal excretion of the test dose observed in the animals given riboflavin every 4 days may have resulted from less efficient absorption of the vitamin under these circumstances. However, Sure and Ford (11) concluded that the rat can efficiently absorb doses of riboflavin much higher than those given in the present study. It would appear more likely, therefore, that the higher fecal riboflavin levels observed may have resulted from excretion of the vitamin into the gastrointestinal tract. Evidence suggesting that this may occur was obtained by Luckey *et al.* (12), who reported that germ-free chicks given a riboflavin-free diet excreted appreciable quantities of riboflavin into the intestine. In recent studies on the mechanism of riboflavin absorption, Middleton *et al.* (13) found evidence indicating that C^{14}-labelled riboflavin was excreted into the intestine of the rat by way of the bile.

The total amount of C^{14}-labelled riboflavin recovered from the tissues, urine, and feces was only a fraction of that given. It would be of great interest, in this connection, to determine the $C^{14}O_2$ content of expired air after giving doses of labelled riboflavin, and such studies are contemplated.

Experiment 4

The results of experiment 4 (Table III) showed that the apparent efficiency of utilization of riboflavin given at 4-day intervals varied with the size of the dose. At the lower doses, the efficiency of utilization was approximately 40%

TABLE III

Efficiency of utilization of riboflavin given at 4-day intervals to growing rats

Riboflavin administered	Dose,* μg/100 g diet	No. of animals	Weight gain, g	Food intake, g
In diet	50	10	6±1.9†	57
By stomach tube	125	9	9±2.5	59
In diet	100	10	19±1.8	68
By stomach tube	250	10	15±1.5	67
In diet	200	10	34±1.4	85
By stomach tube	500	9	19±1.6	71

*Dose for animals given riboflavin by stomach tube at 4-day intervals based on amount of food eaten by pair mate given riboflavin in diet.
†Standard error of the mean.

of that found with riboflavin given in the diet, as evidenced by the fact that the weight gains found with animals given 50 or 100 μg of riboflavin per 100 g diet were not significantly different from those found with animals given 2.5 times these amounts of the vitamin at 4-day intervals. However, the animals given riboflavin in the diet at the level of 200 μg/100 g of diet grew significantly more rapidly than those given 2.5 times this amount of the vitamin at 4-day intervals. At the highest dose, the efficiency of utilization of riboflavin given every 4 days was only approximately 20% of that given daily in the diet. The results with this dose have since been confirmed in another study.

It may be concluded that riboflavin given in a single daily dose is as well utilized by the rat as that consumed with the diet in several small doses over a 24-hour period. The efficiency of utilization is decreased, however, when the doses are given less frequently than daily.

Acknowledgments

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THE CONVERSION OF 2,6-DIAMINOPIMELIC ACID-1,7-C^{14} TO LYSINE-1-C^{14} BY CERTAIN BACTERIA[1]

A. J. FINLAYSON AND F. J. SIMPSON

Abstract

When 2,6-diaminopimelic acid-1,7-C^{14} was added to growing cultures of *Bacillus megaterium*, *Staphlococcus aureus*, and *Escherichia coli*, 8–9% of added carbon-14 appeared in the cellular lysine. Similar experiments with *Proteus vulgaris*, *Streptomyces griseus*, *Aspergillus flavus*, and *Lactobacillus arabinosus* resulted in less than 0.3% of the added carbon-14 being incorporated into the cellular lysine. *Leuconostoc mesenteroides* converted 0.6% of the added DAP-1,7-C^{14} to lysine-1-C^{14}.

Over 90% of the carbon-14 in cell lysine from *B. megaterium* and *L. mesenteroides* was found in the carboxyl carbon. This was interpreted as indicating a direct decarboxylation of DAP-1,7-C^{14} to lysine-1-C^{14}. About 70% of the carbon-14 in the lysine from cells of *S. aureus* and *E. coli* was found in the carboxyl carbon, thus suggesting that some lysine comes from sources other than 2,6-diaminopimelic acid.

Those organisms that actively decarboxylated DAP-1,7-C^{14} to form lysine-C^{14} also synthesized DAP and excreted it into the culture medium during growth.

Introduction

There is much evidence that DAP* found in a number of bacteria by Work *et al.* (1) is a direct precursor of lysine (2). Many organisms containing DAP also possess a diaminopimelic acid decarboxylase that yields 1 mole of carbon dioxide per mole of DAP consumed (3). In addition, mutants of *Escherichia coli* lacking the decarboxylase accumulate DAP (4). Meadow and Work, however, have obtained some evidence that DAP is not the only source of lysine in *E. coli* (5).

Perry and Foster have also suggested that DAP may be a precursor of PDA in spores of *Bacillus cereus* (6) but subsequent studies with *Bacillus megaterium* indicated that PDA more likely arose from the condensation of 3-carbon and 4-carbon fragments.

The synthesis of DAP-1,7-C^{14} in this laboratory has made possible a test for these pathways in microorganisms, by determining if DAP-1,7-C^{14} supplied in the medium is absorbed by the cells, by measuring the amount of lysine-1-C^{14} formed by decarboxylation of DAP, and by measuring the amount of C^{14} found in PDA.

Materials and Methods

(A) Synthesis of 2,6-Diaminopimelic Acid-1,7-C^{14} (7)

Pimelic acid-1,7-C^{14} was prepared from 1,5-dibromopentane (0.50 g, 0.002 mole) and potassium cyanide-C^{14} (0.248 g, 0.004 mole, 0.67 millicurie) by the method of Loftfield (8). The yield of pimelic acid-1,7-C^{14} was 0.180 g (50%).

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*The following abbreviations are employed: DAP, 2,6-diaminopimelic acid; PDA, pyridine-2,6-dicarboxylic acid.

Pimelic acid-1,7-C^{14} (0.180 g, 0.0011 mole) was dissolved in redistilled thionyl chloride (0.500 g, 0.0040 mole) and the solution was warmed on a steam bath until the evolution of sulphur dioxide ceased. Dried bromine (0.480 g, 0.0030 mole) was added to the cooled solution over a period of ½ hour and then the reaction mixture was warmed to 60° C on a steam bath for an additional hour. The solution was cooled and poured into 15 ml of absolute ethanol. Twenty-five milliliters of water was added and the mixture was extracted with three 50-ml portions of ether. The extracts were combined, washed with a 1% sodium sulphite solution, and dried. Evaporation of the solvents left a yellow oil (presumably diethyldibromopimelate-1,7-C^{14}) which was not further purified.

The oil was dissolved in 3.0 ml of ethanol (distilled from magnesium ethoxide) containing freshly cut sodium (0.075 g, 0.0030 mole). Recrystallized succinimide (0.250 g, 0.0025 mole) and a crystal of potassium iodide were then added and the solution was refluxed for 4 hours. After the reaction mixture had cooled, the ethanol was evaporated in a stream of air, and the resulting oil was suspended in 10 ml of 8 *N* hydrochloric acid. The mixture was refluxed 6 hours, then cooled and evaporated to dryness. The solid was taken up in water and the amino acids adsorbed on Dowex 50×8 (H^+ form). The resin was washed with water until the effluent was neutral and the adsorbed amino acids were then eluted with 1.5 *N* ammonia.

Two amino acids were isolated from the ammonia eluate, 2,6-diaminopimelic acid-1,7-C^{14} (0.020 g) and 2-aminopimelic acid-1,7-C^{14} (0.015 g), and were separated by cellulose column chromatography using *n*-butanol:acetic acid:water (4:1:5) as developer. They were chromatographically indistinguishable from their analytically pure non-radioactive counterparts, and in each case the radioactivity corresponded to the ninhydrin spot on paper chromatograms (*n*-butanol:acetic acid:water, 4:1:5, and pyridine:water, 4:1).

Carrier 2,6-diaminopimelic acid was added to the radioactive diaminopimelic acid fraction and 0.035 g of the amino acid was recovered (radiochemical yield, 4%). Its specific activity was 0.75 μc/mg. No attempt was made to separate the isomers of DAP-1,7-C^{14} before use.

(B) Microbiological

B. megaterium, PRL B78, was maintained and grown on a synthetic medium containing $(NH_4)_2SO_4$ (3.0 g), $Na_2HPO_4.12H_2O$ (6.0 g), KH_2PO_4 (3.0 g), NaCl (3.0 g), $MgCl_2.6H_2O$ (0.2 g), $MnCl_2.4H_2O$ (0.005 g), $CaCl_2.2H_2O$ (0.05 g), $ZnSO_4.7H_2O$ (0.005 g), $FeSO_4.7H_2O$ (0.005 g), $CuSO_4.5H_2O$ (0.0001 g), citric acid (0.050 g), and glucose (sterilized separately) (5.0 g/liter). The inoculum was prepared by transferring the culture from an agar slant to 25 ml of medium containing 1 mg of DAP in an Erlenmeyer flask (250 ml) and incubating for 39 hours at 30° C on a rotary shaker. The culture at this time consisted mainly of spores. The flask was held in a water bath at 65° C for 15 minutes, then cooled after which 0.5 ml was used to inoculate 300 ml of test medium.

Lactobacillus arabinosus PRL L4 and *Leuconostoc mesenteroides* PRL L33

were grown on lysine assay medium (Difco) at half strength. Although the organisms grew in the absence of lysine, a small amount (1 μg/ml) was added to encourage heavier growth. Davis' medium containing 0.5% glucose and supplemented with 0.05% yeast extract was used for growing *E. coli* PRL R26 and *Proteus vulgaris* PRL R9 (9). In addition to the yeast extract, Davis' medium was supplemented with 0.05% lysine assay medium (Difco) for growing *Staphlococcus aureus* PRL M2. *Streptomyces griseus* was grown on Saunders' synthetic medium with 1% starch (10) and *Aspergillus flavus* in a medium previously described with 0.5% glucose as the carbohydrate (11).

The ability of lactobacilli to use DAP as a precursor of lysine was tested by growing the organisms in 300 ml of medium in a 1-liter Erlenmeyer flask fitted with a 2-mm bore stopcock at the top and a side arm sealed with a serum bottle cap (12). The basal medium was sterilized in the flask and the DAP-1,7-C^{14} (usually 3 μg per ml of medium) and 0.5 ml of inoculum added aseptically. The flask was then sealed, evacuated to 0.5 atmosphere, and incubated at 30° C for 60 hours.

Essentially the same technique was employed to test the aerobes for the utilization of DAP except that the flasks were fitted with an additional 2-mm bore stopcock about 6 cm from the bottom of the flask. Through this stopcock, sterile air, carbon dioxide free, was blown into the flask. The effluent gases were passed through the stopcock at the top of the flask and through two bead towers, each containing 20 ml of 2 *N* NaOH, and finally through a dry ice trap and a soda lime tube. The fermentation flask was shaken at 100 r.p.m. on a rotary shaker, thus ensuring adequate aeration. *E. coli*, *P. vulgaris*, and *S. aureus* were incubated at 30° C for 18 hours while *S. griseus* and *A. flavus* were grown for 48 hours.

(C) Analytical

When growth was complete the flasks were flushed with nitrogen (carbon dioxide free) to transfer completely the respired carbon dioxide to the alkali in the bead towers. The cells were recovered by centrifugation, washed three times with physiological saline solution, and the washings combined with the culture liquors. The radioactivity contained in the cells, in the respired carbon dioxide, and in the culture liquors was measured (Table I).

The washed cells or spores were hydrolyzed with 6 *N* HCl by refluxing for 24 hours. The amino acids in the hydrolyzate were adsorbed on Dowex 50×8 resin (H^+ form) after evaporation of the excess hydrochloric acid. The carbohydrate and acidic materials were washed through the ion-exchange column with water and then the amino acids eluted with 4 *N* ammonia. The culture media in each experiment were evaporated to dryness and the amino acids present isolated by adsorption on Dowex 50×8 (H^+ form) followed by their elution with 4 *N* ammonia after the neutral and acidic materials had been washed through the resin by water.

Lysine and arginine were separated from the other amino acids by their

selective adsorption on IRC-50 resin buffered with 1 *M* barium acetate (13). They were eluted from the resin with 4% ammonia after the neutral and acidic amino acids had been washed through the column by water.

Lysine was separated from arginine and diaminopimelic acid was separated from the neutral and acidic amino acids by partition chromatography on cellulose using *n*-butanol:acetic acid:water (4:1:5) as the developer. The cells and culture medium in each experiment were examined for lysine and diaminopimelic acid. In addition, glutamic and aspartic acids, valine, leucine, and alanine were isolated from the spores and culture medium of *B. megaterium*. The quantities of the amino acids isolated were determined by the ninhydrin technique of Moore and Stein (14).

PDA was isolated from *B. megaterium* by chromatography of the spore hydrolyzate on Dowex 1×8 (OAc^- form) using 0.5 *N* acetic acid as eluant (15). Its presence was detected in the eluate by measurement of the ultraviolet absorption at 280 mμ of each of the effluent fractions. A ninhydrin negative substance was recovered which had an ultraviolet absorption spectrum indistinguishable with that of a synthetic sample of pyridine-2,6-dicarboxylic acid. Washing the Dowex 1×8 column with 1 *N* hydrochloric acid (15) did not elute any ultraviolet-absorbing material.

The purity of the isolated lysine-C^{14} and DAP-C^{14} was established by isotope dilution techniques using L-lysine hydrochloride and DAP, respectively, as carriers. The specific activities of the amino acids isolated were determined by combusting the samples to carbon dioxide and measuring the radioactivity of the gas in a vibrating reed electrometer (Dynacon, Nuclear Chicago Corp.). Ninhydrin decarboxylation was employed to convert the carboxyl carbon of lysine to carbon dioxide (16).

Results and Discussion

B. megaterium, *S. aureus*, and *E. coli* actively removed carbon-14 from the medium, at least 34 mμc, and had specific activities 13 to 300 times greater than the other organisms tested (Table I). The same three organisms actively metabolized DAP-1,7-C^{14} producing more than 20 mμc of $C^{14}O_2$ and converting the DAP-1,7-C^{14} to lysine-1-C^{14} (Tables I and II). *E. coli* and *S. aureus* have been shown to possess diaminopimelic acid decarboxylase (17).

The lysine in the cells of *B. megaterium*, *S. aureus*, and *E. coli* represented between 8 and 9% of the total C^{14} added to the medium, whereas the lysine in the cells of the other organisms tested represented less than 0.6% (Table II). In *B. megaterium* the lysine had a specific activity 13 times that of any other amino acid isolated except DAP. The specific activity of the lysine in the cells of *B. megaterium*, *S. aureus*, and *E. coli* was much higher than in the other organisms and was 7 to 15 times that of the cells from which it was isolated (Table III).

The data obtained by ninhydrin decarboxylation showed that the lysine isolated from *B. megaterium* and *L. mesenteroides* had about 95% of the carbon-14

TABLE I

Utilization of diaminopimelic acid-1,7-C^{14} by bacteria

Organism	Cells		Respired CO_2		Culture medium
	C^{14} content (mμc)	Specific activity (mμc/g)	C^{14} content (mμc)	Specific activity (mμc/g)	C^{14} content (mμc)
B. megaterium	92.3	507.0	144.0	55.0	278.0
S. aureus	34.0	540.0	34.7	40.0	124.0
E. coli	98.5	142.0	23.5	7.5	103.0
P. vulgaris	3.4	11.0	25.8	12.5	348.0
L. mesenteroides	5.2	51.0	7.3	2.2	267.0
L. arabinosus	2.2	7.1	0.5	4.0	279.0
S. griseus	17.1	10.0	3.1	0.6	276.0
A. flavus	7.7	2.3	2.2	0.8	334.0

NOTE: Ninety-two to a hundred per cent of the added C^{14} was recovered except in experiments with *S. aureus*, *P. vulgaris*, and *A. flavus* where recoveries ranged from 112 to 124%. These organisms were tested after the stock solution of DAP-1,7-C^{14} had been stored for 2 weeks. Evaporation concentrated the stock solution and introduced an error in the calculated amount of tracer used.

TABLE II

Distribution of carbon-14 in cell lysine and diaminopimelic acid

Organism	% of cell C^{14}		% of total C^{14} added
	Lysine-C^{14}	DAP-C^{14}	Lysine
B. megaterium	52*	30	8.4*
S. aureus	52	0	8.2
E. coli	22	10	9.6
P. vulgaris	47	—	0.2
L. mesenteroides	27	0	0.6
L. arabinosus	28	45	0.2
S. griseus	5	7	0.3
A. flavus	11	0	0.3

*Of the C^{14} in the cells of *B. megaterium*, 52% was present as lysine plus arginine, 30% as DAP, and about 1% as PDA. The separation of these amino acids resulted in losses of lysine. These figures are given to show that the amount of DAP converted to lysine was much greater than the amount converted to PDA.

TABLE III

Specific activities* of lysine and diaminopimelic acid

Organism	Lysine		DAP	
	Culture medium	Cells	Culture medium	Cells
B. megaterium	52.5	123.0	280.0	58.0
S. aureus	8.0	169.0	425.0	—
E. coli	17.5	28.5	585.0	113.0
P. vulgaris	9.1	10.0	388.0	2.0
L. mesenteroides	—	14.6	381.0	—
L. arabinosus	—	8.0	2740.0	12.0
S. griseus	—	3.2	8800.0	3.2
A. flavus	—	10.0	6200.0	—

*Specific activities calculated as μc/mole of CO_2 obtained on combustion. Tracer: 2,6-diaminopimelic acid-1,7-C^{14}, 9.2 mc/mole CO_2.

in the carboxyl carbon, demonstrating a direct decarboxylation of DAP-1,7-C^{14} to lysine-1-C^{14} (Table IV). The carboxyl carbon of the lysine from *S. aureus* and *E. coli* contained about 70% of the carbon-14 in the molecule indicating that 30% of the radioactive lysine did not arise from the direct decarboxylation of the added DAP-1,7-C^{14}. The specific activity of carbon dioxide obtained by the enzymatic decarboxylation of lysine from *B. megaterium* was equal to that obtained by ninhydrin decarboxylation (Table IV).

TABLE IV

Carbon-14 content of the carboxyl carbon of lysine

Organism	Specific activity of carboxyl carbon of lysine (μc/mole CO_2)		% C^{14} in carboxyl carbon	% of C^{14} in cells
	Calculated	Found		
B. megaterium	738.0	690.0 (670.0)*	93	48
S. aureus	1040.0	730.0	70	35
E. coli	174.0	114.0	67	15
L. mesenteroides	87.5	83.0	95	26

*Specific activity of carboxyl carbon of lysine obtained through the enzymatic decarboxylation of lysine by the lysine decarboxylase of *B. cadaveris* NCTC 6578 (20).

L. mesenteroides also converted a small proportion of the added DAP to lysine. *B. megaterium* and *L. mesenteroides* apparently converted the DAP-1,7-C^{14} directly to lysine-1-C^{14} and thus apparently have diaminopimelic acid decarboxylase activity. *L. mesenteroides*, however, has been reported not to contain this enzyme (2), but the decarboxylase activity may have been weak and the amount of carbon dioxide released by the enzyme too low to be measured accurately. Certainly, of the DAP-1,7-C^{14} supplied, the amount converted to lysine by *L. mesenteroides* is small compared with *B. megaterium*, but this difference could be attributed to cell permeability rather than decarboxylase activity. *L. mesenteroides*, like *S. aureus*, does not accumulate DAP or deposit it in the cell walls but both, like *B. megaterium* and *E. coli*, are capable of synthesizing DAP and excreting it into the medium (Table III). The specific activities of DAP recovered from the medium on which these four organisms grew showed a 10- to 24-fold dilution by unlabelled DAP.

Whereas *B. megaterium* and *L. mesenteroides* apparently converted the DAP-1,7-C^{14} absorbed directly to lysine-1-C^{14}, *E. coli* and *S. aureus* did not; only 70% of the C^{14} resided in the carboxyl carbon of lysine. A possible explanation is that radioactive metabolites of DAP-1,7-C^{14} or lysine-1-C^{14} may be used by these two organisms to synthesize more DAP-C^{14} which would no longer be labelled exclusively in the carboxyl carbons. Decarboxylation of this material would yield lysine-C^{14} labelled in carbons other than C-1. In support of this hypothesis, the carboxyl carbons of the DAP from cells of *E. coli* contained only 75% of the carbon-14 of the molecule. An alternative explanation may be found in previous reports (5), which have suggested that *E. coli* may possess alternate routes to lysine not involving DAP.

P. vulgaris, while not incorporating as much carbon-14 into its cells, was as active as *E. coli* in decarboxylating DAP-1,7-C^{14} to $C^{14}O_2$. The organism absorbed 0.8% of the carbon-14 supplied in the medium (Table I) and about one-half of this was found in lysine. The data show (Tables I and III) that *P. vulgaris* converted DAP-1,7-C^{14} to lysine-C^{14} to a very small extent, even though at least 7% of the added DAP-1,7-C^{14} was metabolized. Furthermore, the DAP-1,7-C^{14} in the medium had been diluted 20-fold with non-radioactive DAP, demonstrating that *P. vulgaris* synthesized and excreted DAP during growth (Table III). The organism did not absorb exogenous DAP into the cells under the conditions of this experiment. Since Dewey could not detect a DAP decarboxylation in *P. vulgaris* (17) and because the cell lysine had a higher specific activity than the cell DAP, the cell lysine may have been synthesized from carbon-14 containing metabolites of DAP-1,7-C^{14} and not derived by direct decarboxylation.

As *P. vulgaris*, *L. arabinosus*, and *S. griseus* absorbed little DAP-1,7-C^{14} from the medium, one cannot decide whether they do or do not possess diaminopimelic acid decarboxylase, even though the lysine and the DAP in the cells had similar specific activities. *S. griseus*, although containing DAP and capable of synthesizing the amino acid, neither absorbed nor excreted a significant amount. This streptomycete apparently lacks the ability to transport DAP across the cell wall. *L. arabinosus* likewise did not absorb or excrete much DAP even though it did synthesize and accumulate the amino acid (Table II).

No DAP other than that added was detected in the cells or culture medium of *A. flavus*. This, coupled with low utilization of DAP and low specific activity of cell lysine (Table III), agrees with published data for other molds (18), where DAP is not considered to be a precursor of lysine.

Pyridine-2,6-dicarboxylic acid, isolated from the nutrient medium and spore hydrolyzate of *B. megaterium*, did not have a specific activity (10.0 μc/mole of CO_2) significantly greater than glutamic acid (7.3 μc/mole CO_2) or alanine (10.4 μc/mole CO_2), amino acids for which DAP is not considered to be a precursor. In *B. megaterium*, DAP is not converted to PDA as extensively as it is to lysine. This agrees with the results of Martin and Foster, who concluded that DAP is not a precursor of PDA, as was previously proposed (19).

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STUDIES ON THE METABOLISM OF VALERATE-1-C^{14} BY UREDOSPORES OF WHEAT STEM RUST[1]

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Abstract

When uredospores of *Puccinia graminis* var. *tritici* race 15B were shaken in a medium containing *M*/30 phosphate buffer, pH 6.2, and valerate-1-C^{14}, 97% of the radioactivity was removed from the solution in a period of 3 hours. Fifty-five per cent of the carbon-14 was released as carbon dioxide, and 42% was incorporated into the spores. Carbon-14 was found in many cellular components but the water-soluble fraction accounted for 48% of the tracer in the spores. About two thirds of the water-soluble carbon-14 was in a fraction containing amino acids, amides, and peptides, with glutamic acid, glutamine, and γ-aminobutyric acid being highly radioactive. Carbon-5 of glutamic acid and carbon-1 of γ-aminobutyric acid were particularly radioactive. In addition carbon-1 of glutamic acid was appreciably radioactive. The results are consistent with the view that γ-aminobutyric acid was formed by decarboxylation of glutamic acid and that glutamic acid became labelled as a result of β-oxidation of the valerate-1-C^{14} to yield acetate-1-C^{14} which in turn was metabolized by the tricarboxylic acid cycle.

Previous attempts in this laboratory to examine metabolic pathways in wheat stem rust uredospores by the use of exogenous radiotracers were discontinued because of low incorporation into cellular constituents (1, 2). These experiments were confined primarily to studies on utilization of labelled sugars, sugar alcohols, and amino acids. Recent work on respiratory processes of spores has shown, however, that short-chain fatty acids, when externally supplied, have a pronounced effect on respiration rate (3, 4) and are effectively incorporated into the spores (4). It seemed, therefore, that labelled fatty acids would be advantageous in studies on the biosynthetic pathways present in the spores.

The introduction of higher amounts of carbon-14 into the metabolic pools of spores would permit a more adequate study of the operation of the tricarboxylic acid cycle. At present its role in rust spores is by no means clear. Staples and Weinstein (5), for example, suggest that the tricarboxylic acid cycle functions in endogenous metabolism. They feel, however, that its contribution may not be great. Allen (6) concludes, from data collected by Shaw and co-workers (7), that the preferential metabolism is decarboxylation of sugars and that carbon dioxide production via the tricarboxylic cycle is minor. Shaw (8), however, in considering metabolic changes accompanying rust infection of leaves, points out that an increase in carbon dioxide evolution via the pentose phosphate pathway is not necessarily incompatible with a simultaneous increase in the absolute turnover rate of the tricarboxylic acid cycle. Farkas and Ledingham

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(3), in considering conditions of self-inhibition, present data suggesting that respiration by rust spores might occur by fatty acid oxidation pathways that bypass the tricarboxylic acid cycle.

The present communication describes efforts to detect certain metabolic processes in rust spores by analysis of spores after contact with dilute buffered solutions of valerate-1-C^{14}. Valerate was chosen because earlier work indicated that it was most effectively incorporated into the spores. It was used at high specific activity and very low concentrations to obtain maximum substrate conversion.

Materials and Methods

Production of Spores

Uredospores of wheat stem rust (race 15B) were obtained from Stewart wheat plants cultivated in subirrigated gravel culture using Hoagland's solution. A combination of incandescent and fluorescent light provided about 1500 ft-c of light for 18 hours each day. Day temperature was 20° C, night temperature 18° C. Plants were infected with rust spores when 20–30 cm in height, and newly formed spores in sufficient quantity for harvesting usually formed in about 10 days. They were harvested with a vacuum collector (9) and stored at 4° C. Inoculations were repeated at 10-day intervals to assure a constant supply of spores and those more than 14 days of age were discarded.

Labelling of Spores

Fresh spores were incubated in cylindrical dishes 18 cm in diameter and 9 cm in depth, which were provided with a center well 3½ cm in diameter and 4 cm in depth. Thirty milliliters of *M*/30 phosphate buffer, pH 6.2, in which was dissolved 1.7 μmoles of valerate-1-C^{14} (2.0 μc), was added to the outer chamber and 250 mg of spores was floated on the surface of this solution. The center well contained 5 ml of 10% KOH and a 2 in. × 3 in. filter paper wick. A filter paper sheet wetted with buffer was fitted to the interior of the wall of the vessel. The vessel was covered with a rubber sheet and shaken at the rate of 200 r.p.m. on a rotary shaker with a 1-in. radius of motion for a period of 3 hours. The spores were then collected by filtration, thoroughly washed with water, and killed in boiling 40% ethanol. The carbon-14 in the buffer solution and in the alkaline solution from the center well was measured to estimate the radioactivity not utilized by the spores and the radioactivity respired as carbon dioxide respectively. Spores from 12 vessels were pooled for use in experiments described.

Fractionation of Spores

The spores (3 g) were suspended in 30–50 ml of 80% ethanol and ground at 1° C in a planetary mill until completely disintegrated as indicated by microscopic examination (12 hours). The mixture was centrifuged and the residue washed 5 times with 50-ml portions of 80% ethanol and extracted 3 times with 50-ml portions of hot water. The extracts were combined, reduced to dryness, and taken up in 30 ml water. The resulting turbid suspension was clarified by

centrifugation and the residue saved. The aqueous solution was passed through a Dowex-50 column (1 cm diameter × 12 cm long) in the hydrogen form. The column was washed with 100 ml of water and the acid and neutral material collected. An amino acid fraction was then obtained by elution with 0.4 *N* NH_4OH until the effluent was basic. Further elution with 20 ml of 4 *N* NH_4OH was done to insure removal of basic amino acids. The materials not adsorbed on Dowex-50 were placed on a Dowex-1 column (1 cm × 10 cm) in the formate form and washed with 100 ml of water. The effluent comprised the non-ionic fraction such as sugars. Organic acids were eluted with 6 *N* formic acid. The three fractions, i.e. the amino acids and amides, the organic acids, and the non-ionic materials, were evaporated to dryness and aliquots taken for carbon-14 analyses.

Free amino acids and amides were separated on ion-exchange columns using methods of Hirs, Moore, and Stein (10). The acid amides, glutamine and asparagine, were not adsorbed on the Dowex-1 column as were glutamic acid and aspartic acid, but were collected with the "mixture". This mixture was therefore refluxed with 2 *N* HCl for 3 hours to hydrolyze the amides and again chromatographed on Dowex-1. Material originally present as glutamine was thus isolated as a second fraction of glutamic acid. The identity of the amino acids was checked by paper chromatography, their quantity determined colorimetrically, and, after addition of carrier, they were recrystallized and analyzed for carbon-14.

The organic acids were fractionated on a Dowex-1 column (1 cm × 10 cm) by gradient elution with formic acid (1 *N* → 4 *N*) (11). Two-milliliter fractions were collected and the formate removed by evaporation to dryness in a water bath at 50° C. The acids present were estimated by titration with sodium hydroxide and their identity established by paper chromatography (12).

Spore residues were freeze-dried and a 1-g sample hydrolyzed for 24 hours by refluxing with 6 *N* HCl. Amino acids were isolated from the hydrolyzate as described above.

Experimental Results and Discussion

About 97% of the carbon-14 had been removed from the medium upon which the spores were floated in the 3 hours of incubation (Fig. 1). This represents an effective uptake of tracer, and indicates that conditions chosen were suitable for investigations of the type proposed, at least in so far as the substrate valerate is concerned. About 55% of the carbon-14 was released as carbon dioxide and 42% remained in the spores. Much of the carbon-14 in the spores was in the water-soluble material, but the ethanol-soluble substances and the insoluble residue each contained appreciable carbon-14. Thus, not only was valerate carboxyl carbon extensively converted to carbon dioxide but it was also incorporated into a variety of cellular components. The amino acid, amide, and peptide fraction and the organic acid fraction as isolated from water-soluble material were most radioactive and were chosen for more detailed examination.

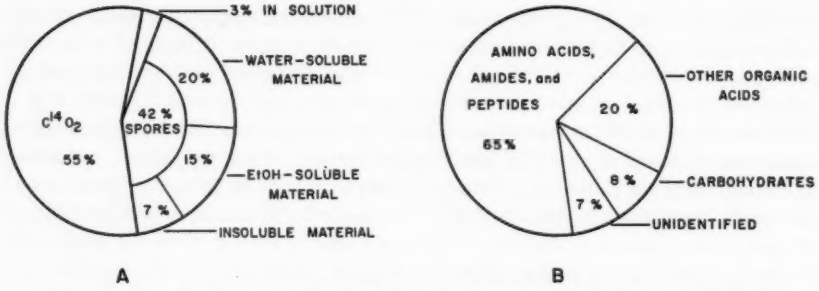

FIG. 1. Distribution of carbon-14 after incubation of rust spores on a buffered solution of valerate-1-C^{14} for 3 hours. (A) Distribution of total amount of carbon-14 originally in buffer. (B) Distribution of carbon-14 present in H_2O-soluble spore material.

Further studies on the labelling of soluble carbohydrates were also done and will be reported elsewhere.[3]

Glutamic acid, glutamine, and γ-aminobutyric acid were similarly labelled and were the most active amino acids isolated (Fig. 2). γ-Aminobutyric acid is not encountered in amino acid mixtures (protein hydrolyzates) ordinarily

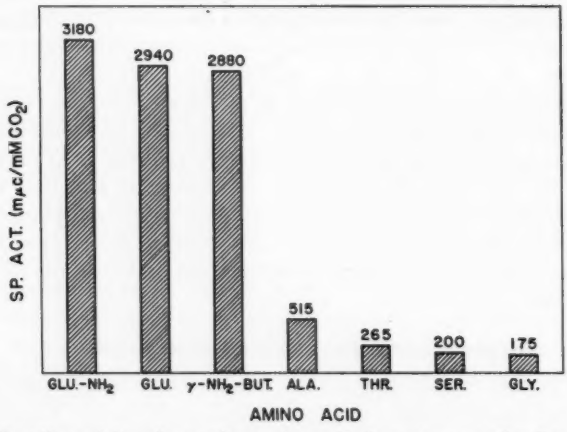

FIG. 2. Specific activity of some free amino acids in rust spores after incubation with valerate-1-C^{14}.
GLU.-NH_2 = glutamine; GLU. = glutamic acid; γ-NH_2-BUT. = γ-aminobutyric acid; ALA. = alanine; THR. = threonine; SER. = serine; GLY. = glycine.

analyzed in this laboratory. The identity of this amino acid was therefore confirmed by recrystallizing it in a large excess of authentic non-radioactive γ-aminobutyric acid. The specific activity of the product agreed with that calculated from the dilution made, whereas α-aminobutyric acid, when recrystallized in the presence of the material, was not radioactive.

[3]Unpublished data (H. Reisener).

Four other amino acids were isolated in pure form and in sufficient quantity for carbon-14 measurements. The most radioactive of these was alanine which nevertheless had a specific activity only about one-fifth that of those mentioned above. The others, threonine, serine, and glycine, were even less radioactive, and had specific activities less than one-half that of alanine. Small quantities of other ninhydrin-positive materials which contained some carbon-14 were also detected. The amounts of the amino acids present in the spores are not tabulated because the isolation procedures were not quantitative. The weights of amino acids recovered were, however, in the following proportions: glutamic acid 5.1, glutamine 5.1, γ-aminobutyric acid 1.8, alanine 3.5, threonine 1.6, serine 1.1, and glycine 1.0. Thus glutamic acid and glutamine are not only highly radioactive but are present in appreciable amounts compared to other amino acids. Data reported can be used to calculate that most of the carbon-14 in the amino fraction was in the glutamic acid, glutamine, and γ-aminobutyric acid and thus supports the conclusion that there has been preferential carbon-14 incorporation into these amino acids.

The specific activities of amino acids not extractable from the spore tissues (Table I) were much lower than with the free acids (Fig. 2), but there was a parallelism between the relative specific activities of these bound amino acids

TABLE I

Specific activity of "bound" amino acids in rust spores incubated with valerate-1-C^{14}

Amino acid	Specific activity (μc/mole CO_2)	Amino acid	Specific activity (μc/mole CO_2)
Glutamic acid	213	Leucine	11
Aspartic acid	133	Arginine	9
Threonine	75	Lysine	8
Alanine	57	Valine	4
Glycine	38	Isoleucine	2
Serine	17	Phenylalanine	2

and the free amino acids. Glutamic acid was most radioactive and aspartic acid and threonine were appreciably labelled. Alanine again was one of the more radioactive amino acids. The presence of carbon-14 in the bound amino acids could result from protein biosynthesis in the cell during the incubation period. A "protein" fraction obtained by dilute alkali extraction and precipitation with trichloroacetic acid was radioactive but the preparation could not be regarded as rigorously pure protein. Other possible explanations for radioactivity in bound amino acids are possible and the results cannot be offered as proof for the occurrence of protein biosynthesis during the incubation period.

Arginine and proline are considered to be biosynthetically related to glutamic acid (13) and frequently reflect this relation in tracer experiments by similarity in labelling (14). It is noteworthy therefore that in the present work only a small amount of low specific activity arginine was isolated and that both the quantity and carbon-14 content of proline were too small for estimation.

The specific activity of the carboxyl group of γ-aminobutyric acid and of each carboxyl group in the glutamic acid is shown in Fig. 3. Carbon-1 and carbon-5 together account for about three quarters of the carbon-14 in the

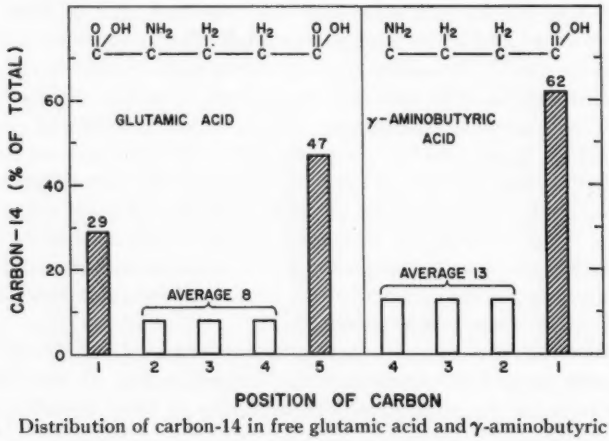

FIG. 3. Distribution of carbon-14 in free glutamic acid and γ-aminobutyric acid.

glutamic acid, with carbon-5 alone accounting for about 47%. The internal carbons were not isolated but by calculation the average carbon-14 content of these is about 8% of the total. This labelling is entirely in agreement with predictions following on the assumptions that valerate-1-C^{14} is extensively oxidized by β-oxidation (15) to yield acetate-1-C^{14} which in turn is metabolized by the tricarboxylic acid cycle. Glutamic acid from α-ketoglutaric acid formed from acetate-1-C^{14} on the first turn of the cycle would be labelled in carbon-5. If, however, recycling occurs carbon-14 would appear in carbon-1. Glutamic acid predominately labelled in position-5 with significant carbon-14 in position-1 is in fact typical of acetate-1-C^{14} feeding in other systems (16, 17), and since we are not aware of alternate mechanisms producing a similar pattern the data are interpreted as evidence that the sequence of events suggested above has occurred. The "high" glutamic acid content suggest that the mechanism was relatively vigorous during the incubation period.

The specificity of carbon-14 incorporation into carbon-1 of γ-aminobutyric acid is evident. The data in Fig. 3 has been arranged to show the similarity of the pattern for carbons 2, 3, 4, and 5 of glutamic acid with the sequence carbons 4, 3, 2, and 1 of γ-aminobutyric acid. The specific activity of carbon-1 of γ-aminobutyric acid was slightly less than that of carbon-5 of glutamate, while the average specific activity of internal glutamate carbons was similar to the average for carbons 2, 3, and 4 of γ-aminobutyric acid. The observations are readily explained by assuming γ-aminobutyric acid formation by decarboxylation of glutamate (18).

The specific activity and yield of three organic acids adequately identified and purified are shown in Table II. The results indicate extensive incorporation of carbon-14 into citrate and in general supports the conclusion that the

TABLE II

Labelling of some organic acids

Acid	Weight isolated (mg)	Specific activity (μc/mole CO_2)
Citric acid	3.7	4,600
Malic acid	0.75	1,900
Succinic acid	0.11	1,300

tricarboxylic acid cycle was involved in metabolism of the valerate. Six other fractions, some clearly radioactive, were also obtained but their identity and purity was not established. Staples and co-workers (19) have reported a more detailed study of organic acids in rust spores and hence one of the primary objectives of the study on this fraction was to indicate incorporation of valerate carboxyl carbon into this group of acids.

Although the present investigation is preliminary in nature and involves only one labelled substrate, it has indicated a technique for introducing adequate amounts of endogenous carbon-14 into the metabolic pools of intact spores and as such provides means for studying the metabolic pathways operative. Data reported was taken from a single experiment using 12 incubation vessels. Exploratory experiments, although somewhat less complete because of analytical difficulties not resolved at the time, gave results entirely consistent in a qualitative sense with those reported.

The data demonstrate that many biological processes occur in spores among which are the biosynthesis of amino acids, organic acids, carbohydrates, and probably protein. More particularly the data has given evidence for β-oxidation of valerate and for the operation of the tricarboxylic acid cycle.

Acknowledgments

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IMMUNOCHEMICAL EVIDENCE FOR A GAMMA GLOBULIN PECULIAR TO CEREBROSPINAL FLUID[1]

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Abstract

Two immunologically different gamma globulins were revealed in "normal" cerebrospinal fluid (NCSF) and in multiple sclerosis cerebrospinal fluid (MSCSF) by immunoelectrophoretic analyses, which employed rabbit antisera prepared against NCSF and MSCSF. Since only one gamma-globulin arc was formed on immunoelectrophoretic analyses of normal human sera (NHS) with rabbit anti-CSF sera and repeated absorptions of anti-CSF sera with human serum failed to remove antibodies to the minor gamma globulin, it would appear that the minor gamma globulin is peculiar to CSF. The major gamma globulin of CSF was shown to be immunologically identical with the single gamma globulin of serum as judged by the fusion of their respective precipitin arcs. The major and minor gamma globulins of NCSF could not be distinguished from those of MSCSF by any of the rabbit antisera used. However, the ends of the precipitin arc formed by the major gamma globulin of NCSF with its homologous antibody became frayed and sometimes doubled indicating it to be immunologically less homogeneous than the gamma globulin of serum. The two gamma globulins of MSCSF and the minor gamma globulin of NCSF migrated more slowly than the major gamma globulin of NCSF. In comparison with NHS, the immunoelectrophoretic patterns of CSF developed with anti-CSF sera were characterized, in the alpha-2 zone, by a greater number of stronger arcs. Experiments on absorbed anti-CSF sera indicated that this was due to differences in the relative concentrations of the various components of this zone in the two fluids.

Introduction

The study of normal human cerebrospinal fluid (NCSF) by immunoelectrophoresis was first undertaken by Gavrilesco and co-workers (1). They were able to find only 12 of the 16 to 18 proteins (2) which could be demonstrated in normal human serum (NHS); the gamma globulin in NCSF was relatively less concentrated and migrated more slowly than that in NHS, while the beta-2 globulin and the rho glycoprotein were relatively more concentrated than in serum; moreover, some of the alpha-1-, alpha-2-, and beta-1-globulin bands appeared to be either very weak or absent. This work, and most of the studies done subsequently, were carried out with antisera prepared in horses by prolonged immunization with NHS. Scheiffarth (3), using a rabbit anti-NHS serum, also found 12 antigens in NCSF. Since specific antisera to pure human serum proteins have become commercially available, transferrin (4, 5, 6), the alpha-1 glycoprotein (4), and the alpha-2 macroglobulin (6) have been detected in NCSF. Indeed about 16 components (6) have now been counted in NCSF as against some 25 in NHS (7).

It has usually been assumed that the antigens revealed in CSF and NHS which have similar mobilities also have identical immunological properties.

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Contribution from the Department of Neurology and Neurosurgery, the Montreal Neurological Institute, McGill University, Montreal, Que. This work was supported in part by grants from the National Multiple Sclerosis Society of Canada and was first presented on June 1, 1961, at the meeting of the Canadian Federation of Biological Societies at Guelph, Ontario.

This assumption has been verified by Frick (4) for the albumins, the alpha-1 glycoproteins, the beta-1 metal-binding protein, and the gamma globulins of the two fluids, using Ouchterlony's (8) criterion of immunological identity. While it is probable that this assumption of immunological identity is correct for the remaining antigens common to the two fluids, the possibility should be borne in mind that many could be merely similar enough to cross react extensively.

In the present investigation a basic immunological principle was followed: the antisera used to analyze CSF were prepared by immunization with CSF. Thus if any substance peculiar to CSF existed, it would be revealed by its homologous antibody. Moreover, interpretation of the results would be free from the doubts which arise whenever possibly heterologous, but extensively cross-reacting, antibodies are used for immunological analyses.

Materials and Methods

Antigens

The normal human serum (NHS) used was a pool of sera obtained from 10 laboratory personel. After the NHS was heated to 56° C for ½ hour and merthiolate was added to a final concentration of 1:10,000, it was divided into 10-ml aliquots and stored at −15° C until needed. Multiple sclerosis serum (MSS) was also a pool of several sera obtained from patients chronically ill with multiple sclerosis.

"Normal" cerebrospinal fluid (NCSF) was drawn from patients having migraine, chronic anxiety states, hysteria, or epileptic seizures for which no anatomical neurological cause could be found. All had normal X rays of the skull, some had dilatation of the ventricular system, but all had normal spinal fluid protein concentration, negative Lange colloidal gold curves, and no increased cell count. Multiple sclerosis cerebrospinal fluid (MSCSF) came from patients who were diagnosed as having multiple sclerosis in the Montreal Neurological Institute. The spinal fluid specimens were frozen at −15° C as soon after collection as possible. When needed, they were thawed, pooled, and concentrated. In the figures, subscripts following NCSF or MSCSF indicate batch numbers of concentrated, pooled, cerebrospinal fluids.

Production and Preparation of Antisera

Rabbit antisera were prepared against NHS, MSS, NCSF, and MSCSF. NHS and MSS were diluted with an equal volume of saline containing 1:5000 merthiolate, and then mixed with an equal volume of Difco Freund adjuvant (complete) to form a stable emulsion. Cerebrospinal fluid antigens were prepared similarly but contained about one-half as much total protein (7 mg/ml). Once a week for 4 to 5 weeks, 1.0 to 3.0 ml of these antigens was injected intramuscularly, one quarter of the dose into each of four sites (9, 10). When an animal had received about 60 mg of cerebrospinal fluid protein or 100 mg of serum protein, it was allowed to rest for 1 month. Most of the antisera were preserved with phenol (0.25%) and merthiolate (1:10,000) and kept frozen at

−15° C until needed. The approximate strengths of the various antisera were determined by adding 0.25 ml of increasingly concentrated solutions of NHS in saline to a constant mixture of 0.25 ml of an antiserum and 0.25 ml of saline. After the reactants had been mixed, the concentration of antigen which produced the most rapid flocculation was noted. If the most rapid flocculation was obtained with at least 1.0 mg NHS protein per ml of antiserum, the serum was considered to have a sufficiently high antibody level and the animal was bled from the ear. After a rest of 1 month, the bleeding was repeated if the serum was still strong. Otherwise, two or more injections of the appropriate antigen were given and the serum was tested again 3 weeks after the last injection. This modified optimal-proportions method was used as an indicator of the relative strengths of the sera, as it is impractical to find the equivalence point of an antiserum to a mixture of antigens such as serum or cerebrospinal fluid.

Antibodies to NHS proteins were absorbed from an aliquot of an anti-NCSF serum by adding an amount of undiluted NHS calculated from previous tests to achieve optimal proportions. The sera were well mixed and allowed to stand at 37° C for ½ hour and then at 4° C overnight. Then the specific precipitate was removed by centrifugation and the supernatant was absorbed with 1/10th the volume of NHS used for the first absorption. On successive absorptions, the mixtures were allowed to stand 2–4 days at 4° C, and the volume of NHS used for absorption was doubled each time, until that volume used for the first absorption was reached. Usually, only small amounts of precipitates formed after the second absorption. The absorptions were carried out in this fashion to avoid the formation of soluble antigen–antibody complexes.

The horse antinormal human serum antiserum was obtained from the Pasteur Institute.

Protein concentration was determined by the sulphosalicylic acid method of Cipriani and Brophy (11).

Cerebrospinal fluid was concentrated 100-fold by ultrafiltration in the cold at 4° C, by the method of Mies (12).

Immunoelectrophoretic analyses were carried out according to Grabar and Williams (2) as modified by Courcon and Uriel (13). Plates were poured using 1.25% washed Difco Bacto agar containing barbiturate buffer at pH. 8.6 and 0.025 μ. Reservoirs of 0.1-ml volume were cut in the agar with a cork borer. About 2.0 mg total protein was used for each separation.

The agar plates were photographed, using dark-field illumination, with a high-contrast film. Photographs of the plates were taken early in the development of the precipitin arcs and, thereafter, whenever some significant change occurred. After full development, the plates were either discarded or stained (13) and photographed again.

For the double diffusion in gel or Ouchterlony type experiments, 1% Difco Bacto agar containing 0.10 μ sodium chloride and 0.05 μ barbiturate buffer, pH 8.6, was poured into Petri dishes to a depth of 4 mm. Circular wells were cut with cork borers in patterns to suit the aims of different experiments.

Results

Rabbits immunized with CSF or NHS according to the method described above usually required a second course of two to three injections of antigens before they developed antisera of the desired strength. Except for the rather low level of antibodies to the beta-1 globulins, antisera to CSF were superior to antisera to NHS in revealing components in either fluid.

When NCSF and NHS were placed in adjacent reservoirs and separated electrophoretically, and the surrounding trenches were then filled with a rabbit anti-NCSF serum, precipitin arcs developed as shown in Fig. 1.

Two immunologically distinct gamma-globulin arcs appeared in the NCSF while only one arc formed in the gamma-globulin region of serum. After 4 to 5 days the heavier arc belonging to the slightly faster gamma globulin of NCSF became frayed at the anodal end and split into two lines at the cathodal end (Fig. 1B). The single gamma-globulin arc of NHS showed no evidence of inhomogeneity, even after 2 weeks. The thinner arc belonging to the slightly slower gamma globulin of cerebrospinal fluid had a tendency to fade after a few days.

In the beta-2 zone of NCSF only the single broad heavy arc characteristic of cerebrospinal fluid was formed while a strong arc and a weak arc were always noted in the corresponding region of NHS. At least two precipitin arcs in the beta-1 regions were formed by both fluids.

In the alpha-2-globulin zone of CSF at least five or six discrete arcs could easily be counted while only two to four arcs could be discerned in the NHS region (Fig. 1B). Although pure proteins were not available to identify the arcs in the alpha-1-and serum-albumin regions, it has been assumed from other studies (14, 15, 16) that the faint arc next to the heavy albumin arc is the alpha-1 globulin of S 3.5. In addition, what is presumably the alpha-X-globulin arc (14) is visible in the CSF region as an offshoot of the alpha-1 arc (Fig. 1B). It will be noted that there was a single arc in the albumin regions of both fluids and these arcs fused (Figs. 1B and 1C) showing immunological identity of the two albumins. When a transverse trench filled with antiserum was placed so as to allow for fusion of the gamma-globulin arcs also, it will be noted from Fig. 1C that the arcs of the sole gamma globulin of NHS and the major gamma globulin of CSF have fused, showing their immunological identity. The line of the second, slower, gamma globulin of CSF simply faded as it reached the serum region indicating that it is unrelated to the serum gamma globulin.

In Fig. 2 are shown results of similar experiments with multiple sclerosis serum and multiple sclerosis spinal fluid in place of the corresponding normal fluids. Antiserum to MSCSF was used in the surrounding trenches. Again the two gamma-globulin arcs appeared in the CSF region and a single gamma-globulin arc in the serum area. The arc of the major gamma globulin of MSCSF fused with the arc of the serum gamma globulin. As before, the line for the minor gamma globulin faded as it approached the area where serum had diffused.

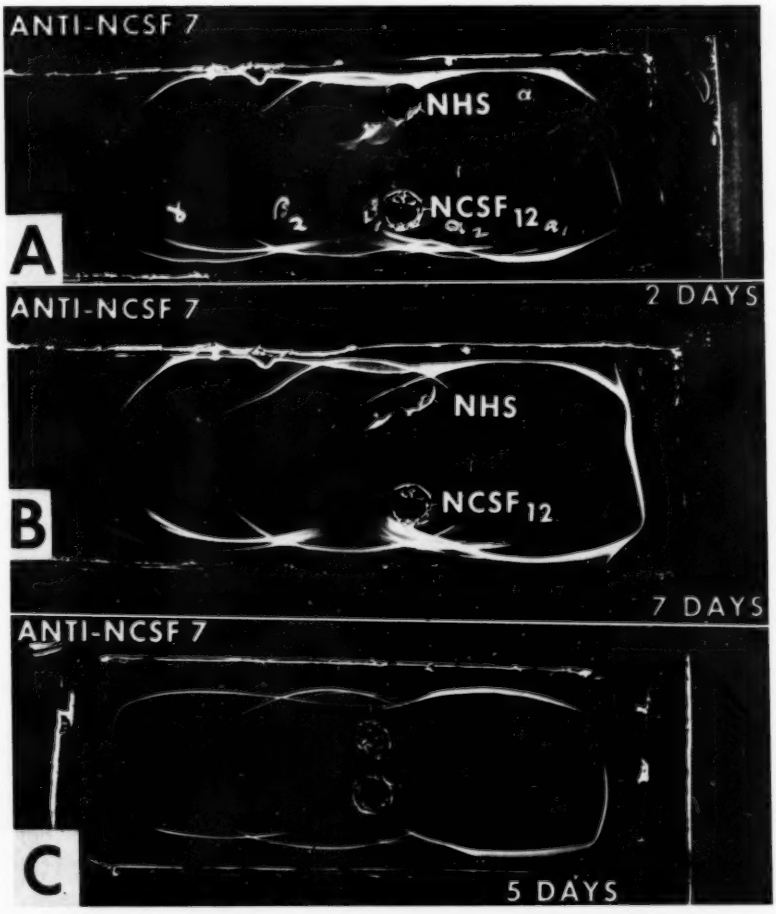

FIG. 1. Immunoelectrophoretic patterns formed by NHS and NCSF with an anti-NCSF serum: (A) after 2 days; (B) after 7 days; (C) photograph of an identical study taken after 5 days, but with a transverse trench for antiserum near the ends of both the gamma-globulin and albumin zones.

PLATE II

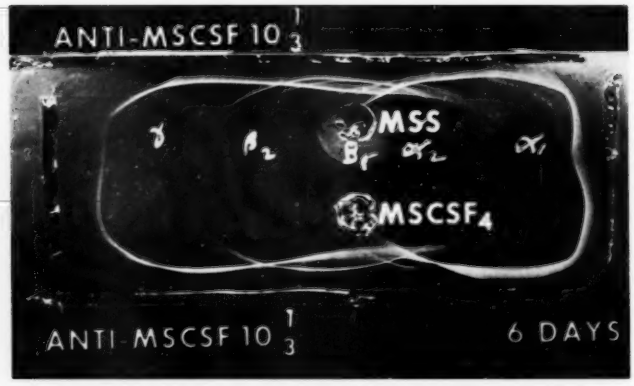

FIG. 2. Immunoelectrophoretic patterns of MSS and MSCSF developed by an anti-MSCSF serum.

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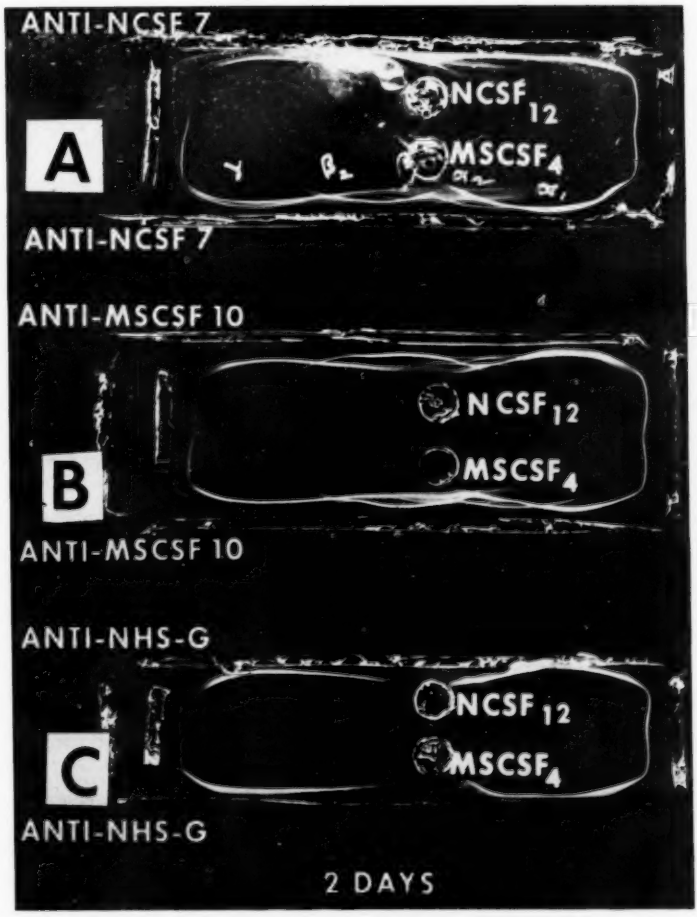

FIG. 3. Immunoelectrophoretic patterns of NCSF and MSCSF developed by (A) an anti-NCSF serum, (B) an anti-MSCSF serum, (C) an anti-NHS serum.

It will be noted that the arcs which are nearest to the fused albumin arcs, and which are presumably formed by the main alpha-1 globulins, have also fused, showing these two components to be identical immunologically. With this antiserum (No. 10) the three well-known arcs in the beta-1-globulin zone of serum were well brought out, while the alpha-2-globulin region of the CSF is again seen to have a larger number of lines than the corresponding area in serum.

It was considered of interest to ascertain whether the major and minor gamma globulins of NCSF and MSCSF were identical by immunological tests. Paired samples of the two kinds of CSF were electrophoretically separated from adjacent reservoirs. The trench around the first pair was filled with an anti-NCSF serum, the trench around the second pair by an anti-MSCSF serum, and the trench around the third pair by a strong anti-NHS serum. It will be noted, from Figs. 3A and 3B, that with the two anti-CSF sera used for analyses, two gamma-globulin arcs developed in both fluids and, moreover, the arcs of the corresponding gamma globulins fused. Thus it appears that with rabbit antibodies, no immunological differences can be shown between the gamma globulins in the CSF of normal people and those with multiple sclerosis.

Only one gamma-globulin arc developed when anti-NHS serum was used to reveal the constituents of cerebrospinal fluid (Fig. 3C). Since antibody only to the single serum gamma globulin exists in this antiserum, the findings with this antiserum agree with results obtained with anti-CSF sera namely that the major gamma globulin of CSF and the gamma globulin of serum are the components common to the two fluids.

Another way to ascertain whether the second or minor gamma globulin of CSF is specific for that fluid, or exists also in serum but in very low concentration, was to attempt to absorb all the antibodies from anti-CSF serum with NHS. Accordingly, anti-NCSF sera were absorbed with NHS following the procedure described above. The completeness of the removal of the antibodies to NHS was followed by ascertaining if the absorbed sera still formed precipitin bands with NHS in a gel diffusion analysis. It was found that antibodies to all but two of the antigens of NHS were removed in the first two absorptions and that additional absorptions had little effect in reducing the concentration of the antibodies which remained.

Patterns of the precipitin bands formed by one anti-NCSF serum absorbed three times, and another anti-NCSF serum absorbed eight times, are illustrated in Fig. 4A.

Figure 4B is a drawing of an immunoelectrophoretic study of NCSF and NHS using the anti-NCSF serum No. 7 absorbed eight times with NHS as the analytical serum. The three precipitin arcs in the NCSF region are shown to belong respectively to the minor gamma globulin and to two alpha-2 globulins, one of the latter proteins forming a blurred line and the other a sharp arc. In the NHS region one indistinct band and one sharp band are found also in the alpha-2 globulin zone. Thus, it would appear that these two alpha-2-

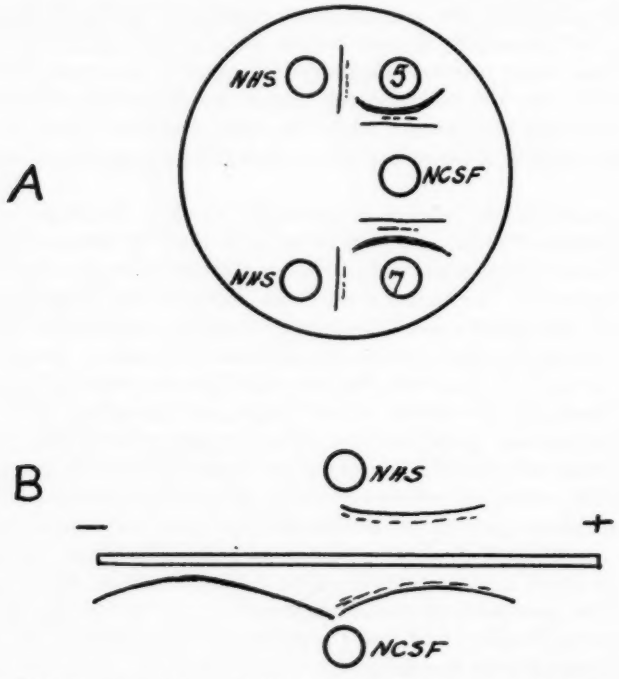

FIG. 4. (A) Diagram of the precipitin lines formed in an agar gel when anti-NCSF serum No. 5 and anti-NCSF No. 7 (absorbed three and eight times respectively with NHS) are allowed to diffuse toward undiluted NHS and NCSF (3.0 mg protein/ml).
(B) Immunoelectrophoretic pattern of NHS concentrated almost 10 times (760 mg protein/ml) compared with that of NCSF (20 mg protein/ml) when developed by anti-NCSF serum No. 7 absorbed eight times with NHS.

globulin antigens exist in serum in such low concentrations that it is impractical to remove antibodies to them by using whole NHS as the absorbing agent.

There was no trace of the minor gamma globulin of CSF in our NHS pool, even when NHS concentrated almost 10-fold was analyzed. Moreover, a concentrated solution (1.6%) of immune gamma globulin prepared by Connaught Laboratories from normal pooled human plasma did not form a line in a gel diffusion analysis with the absorbed anti-NCSF serum containing the antibody to the CSF minor gamma globulin.

Discussion

The present studies show that when cerebrospinal fluid is combined with Freund adjuvant and injected into several sites in the muscles of rabbits it is an excellent antigen for producing precipitating antisera which have a spectrum and concentration of antibodies similar to those elicited by human serum. Indeed, it was found superior to human serum in engendering a higher con-

centration of antibodies to the alpha-2 and beta-2 groups of globulins. The heightened formation of antibodies to the beta-2 globulins can be explained as the response to a greater antigenic stimulus, for the beta-2 globulins are relatively more concentrated in CSF than in NHS. However, paper electrophoresis studies in this laboratory showed that the concentration of alpha-2 globulins in both fluids was about the same and the results of absorption experiments (Fig. 4) indicated that there were no alpha-2 globulins in CSF that could not also be detected in serum. Thus it seems apparent that the concentrations of some of the alpha-2 globulins are much greater in CSF and this results in a higher level of antibody production.

Immunological analyses of CSF and NHS with anti-CSF sera showed that there is a gamma globulin in CSF which is absent from human serum. The antibody to this gamma globulin could not be removed from anti-NCSF sera by exhaustive absorption with pooled NHS. Conversely, the gamma globulin could not be demonstrated in our pooled NHS concentrated 10-fold by ultrafiltration, or in Connaught immune serum gamma globulin prepared from pooled normal human plasma. Antibodies to this protein were also absent from the sera of rabbits hyperimmunized with NHS.

This gamma globulin was found in all cerebrospinal fluids examined and many of these were drawn from patients with widely diverse neurological illnesses. It would appear, then, to be a protein which characterizes CSF. This protein migrated more slowly than the major gamma-globulin constituent of CSF and the position of its precipitin arc close to the antiserum trench suggests it to be a smaller, more rapidly diffusing, molecule than the other gamma globulin. Experiments are now in progress to isolate and characterize this substance more fully. The presence of this gamma globulin peculiar to the CSF confirms the suggestion of Kabat (17) and the view of Katzenelbogen (18) that central nervous tissue is capable of forming gamma globulin. It would be of interest to find out whether any of this particular gamma globulin functions as an antibody.

Whenever the major gamma globulin of CSF and the serum gamma globulin were allowed simultaneously to diffuse toward either anti-NCSF serum or anti-NHS serum, the respective precipitin arcs fused. While the two proteins thus appeared to be immunologically interchangeable, they were not exactly alike. The arcs of the gamma globulins may be seen in almost their entire length in most of the figures and it will be noted from the position and appearance of the arc of the NCSF globulin that this protein had a slightly faster electrophoretic mobility (see Figs. 1 and 3) and was immunologically less homogeneous than the counterpart in serum. These precipitin arcs of the CSF protein usually frayed at the anodal end and after a few days often split into two distinct lines at the cathodal end (Fig. 1A). Other precipitin arcs on the same agar plates did not show these developments and the serum gamma-globulin line never exhibited evidence of doubling. A greater than usual degree of inhomogeneity in the major gamma globulin of a CSF specimen might explain why Chevance

et al. (19) found two gamma-globulin arcs in only one of the samples of CSF they subjected to immunoelectrophoretic analysis using their anti-NCSF rabbit serum (20).

The two gamma globulins in multiple sclerosis spinal fluid were found to be immunologically interchangeable with their counterparts in normal fluid by analysis with rabbit anti-NCSF or anti-MSCSF sera (see Fig. 3). However, the major gamma globulin in MSCSF appears to have a somewhat slower electrophoretic mobility than the major component in NCSF.

While analyses with rabbit antisera could not distinguish the major gamma globulin of NCSF from that of MSCSF, studies with horse anti-NHS sera showed that a marked change had occurred in this MSCSF protein. Like Frick (21), who also used horse anti-NHS sera for immunoelectrophoretic analyses, we found two gamma globulins in the MSCSF pools and only one in the NCSF pools. Thus it is apparent that with horse anti-NHS sera, but not with the homologous rabbit anti-CSF sera, a variation, related to disease, is revealed in the MSCSF gamma globulin which arises from the serum.

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OLFACTORY PERCEPTION IN MIGRATING SALMON

II. STUDIES ON A LABORATORY BIO-ASSAY FOR HOMESTREAM WATER AND MAMMALIAN REPELLENT[1]

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Abstract

A laboratory bio-assay, based on the response of adult migrating sockeye salmon (*Oncorhynchus nerka*) to homestream water, is described. A characteristic response to the repellent in mammalian skin is discussed. The homestream substance(s) was tentatively identified as volatile, dialyzable, neutral, and heat-labile.

The transportation and holding of sockeye in captivity was investigated.

Introduction

Many studies have clearly demonstrated the keen olfactory perception of fish (1, 2, 3, 4, 5, 6). Salmon respond to a repellent in mammalian skin at extremely high dilution (7, 8) and the young eel can be trained to detect 1 part of β-phenyl ethylalcohol in 3×10^{18} parts of water (9).

The ability of salmon species to return to spawn in the stream of their birth is adequately documented and reviewed (10, 11). For example, nearly one-half million sockeye (*Oncorhynchus nerka*) fingerlings were marked and released in a Fraser River stream and 11,000 adults returned to the parent stream but none to nearby tributaries (12).

In another instance, a marked steelhead was released in the Alsea River, Oregon, in April, 1958. The fish was captured and tagged near Alaska on September 5, 1958. It was recovered back in the Alsea River on February 5, 1960. This fish made a minimum round trip of 2400 miles to return to the stream of its birth (13).

An interesting question was examined by Higgins (14). Given a choice, will salmon return to the homestream of their parents or to the water in which they spent their first months of life? Higgins transplanted sockeye eggs from Baker River, a tributary of the Skagit River in Washington, to Grandy Creek, a stream located above Baker River but emptying into Skagit River. The hatched fry from these transplanted eggs were marked. In due course it was noted that marked adults returned only to Grandy Creek. In 1949, scientists of the International Pacific Salmon Fisheries Commission investigated this question further by transporting fertilized sockeye eggs from the Horsefly River and hatching them in the Quesnel Field Station on Horsefly Lake (15). In November, 1950, the fingerlings were transported by air downstream to Quesnel Lake at the mouth of the Horsefly River. In 1953 only one marked fish was

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recovered in the homestream of the parent stock, while 203 marked adults were recovered in the area of the hatchery. Thus, the fish returned to their homestream water by a route which they had never before travelled.

Craige's pioneering study (16) and Wisby and Hasler's (17) experiments lend strong support to a homestream odor theory. In the latter work, sexually ripe coho salmon were captured in their homestream on a branch of the Isaquah River, Washington. They were returned downstream below a fork and the nasal sacs of half of the fish were plugged with cotton while the remainder were untreated. The untreated fish returned again to the stream of their first choice, while the treated fish returned in a random fashion to the two tributaries (17).

The goals of the present studies were to develop laboratory bio-assays to facilitate the isolation and identification of the homestream odor(s) and to permit further study of the salmon repellent in mammalian skin (7, 8).

Methods and Materials

Selection of Fish

In selecting the species of salmon to be used, careful consideration was given to the following factors: the migratory routes of the fish and their accessibility for fish trapping and water sampling; the migratory behavior pattern of the fish and its suitability for study in the limited space of aquaria; the relative inherent resistance of the different species of salmon to disease, i.e. the adaptability and survival of the fish in captivity. Information on the relative resistance of the various species of adult salmon to disease is scant. Experience of fish culturists has indicated that the coho can be held in captivity for periods of up to several months. While the coho and spring salmon show strong territorial behavior, the sockeye, pink, and chum display schooling behavior. The latter behavior pattern would be the more desirable in the confined space of aquaria. While both the pink and the chum vary greatly in their weights and lengths at maturity, the adult sockeye of a given race is uniform in both weight and size. It thus appeared that the salmon species best suited for the study was the sockeye, providing suitable conditions for holding the fish in captivity could be worked out.

The sockeye is abundant along the whole west coast from Alaska to California. The life cycle varies somewhat between localities. Once the yolk sac is absorbed, the fingerling usually spends 1 year in the lake and then migrates to sea during the spring. In certain areas, however, the fingerling may spend 2 or 3 years in fresh water before beginning the seaward migration. Most sockeye mature after having spent 3 to 4 years at sea. While information concerning the movements of sockeye at sea is meager, the migratory routes of these salmon in the major river systems have been studied extensively with the use of tags, traps, and counting fences. In general, the weight of the mature sockeye is between 5 and 7 pounds. Adult sockeye cease to feed when they enter fresh water for their spawning migration. While both sexes are similar in external appearance at the start of the spawning migration, the external appearance of

the male changes dramatically a few weeks prior to spawning. The skin of the male changes in color from a dull grey-green to brilliant red. Also, the male develops a distinct hump and a pronounced hooked snout. The major noticeable external change in the female from the start of the spawning migration to the time of spawning is a loss in the brilliance and coloring of the skin and a lesser elongation of the snout. Sockeye, whether as fry migrating to sea or as adults migrating to the spawning beds, school constantly.

Handling of Fish

In order to minimize bruising and loss of scales while transferring the fish from the trap to the transport tank and to aquaria, only wet wool gloves and knotless nylon nets were used. If the transport distance exceeded 40 feet the fish were carried in a 1 ft × 1 ft × 6 ft aluminum tank filled with water. To avoid injury to the fish during these short transfers, an anaesthetic, methane tricaine sulphonate (MS 222), was added to the water at a level of 1:50,000. This concentration of anaesthetic induced a mild sedation which minimized struggling and subsequent injury to the fish.

Transport

The transport tank used to transfer the fish from the field sites to the aquarium was a circular 400-gallon aluminum tank 4 feet in diameter and 3 feet in height with a conical top. A bilge pump placed at the bottom of the tank gave continuous water circulation at the rate of 3 gallons/minute. The water outlet from the pump, situated at the top of the tank, was fitted with a sieve to disperse the water in a fine spray. This dispersion of water not only aided circulation but also facilitated aeration. As an added precaution to ensure maximum available oxygen and minimum carbon dioxide build-up in the water, the top of the tank was perforated with numerous small holes to allow free air passage in and out of the tank. In addition, oxygen was passed into the water from a pressure cylinder. The water was isotonic with respect to sodium chloride. To placate the fish, MS 222 was added to the water at a level of 1:100,000. This concentration of anaesthetic induced a very mild form of sedation from which the fish recovered quickly when transferred to fresh water. The water temperature during transport was held at 7° to 9° C. During the summer it was found necessary to add ice to the water in order to maintain this temperature. The ice was prepared from chlorine-free water. To minimize the risk of infection, chloromycetin was added at a level of 25 mg per gallon of water. Under these conditions, a total of 250 sockeye were transported distances of up to 250 miles without mortality. The largest single load was 50 fish.

Holding and Experimental Tanks

Two 6 ft × 3 ft circular aquaria constructed of plywood, coated on the inside with fiberglas, and having a 1-ft center core, were used to hold fish which were not under immediate study. At Courtenay, B.C., the fish were held in outdoor concrete ponds (36 ft × 6 ft × 4 ft) with adequate water flow. The experimental aquarium, 14 ft × 3 ft × 3 ft, was constructed of cement and the front plate of

2-in. photographic glass. Each reserve tank had a capacity of 300 gallons and the experimental tank 757 gallons. In order to conserve test materials, the experimental tank contained only 400 gallons of water during use. All water was dechlorinated and carried via iron or plastic pipe. Water was supplied to the reserve tanks at a rate varying between 8 and 10 gallons/minute and to the experimental tank at 3 to 4 gallons/minute. Each tank was aerated with spargers.

Source of Fish

When captured, all fish were actively engaged in spawning migration. The sockeye employed in the initial phase of this study were obtained during July, 1960, at the outlet to Great Central Lake. This water system located in the Alberni area of Vancouver Island, B.C., supports a well-established sockeye population. These fish showed none of the external changes associated with sexual maturity. They normally spawn in late October and November. The second group of fish were obtained during early November at Sweltzer Creek, the outlet to Cultus Lake. This watershed located in the southern interior of British Columbia also supports a sizable sockeye population. These fish when captured were advanced in their sexual development and would normally spawn in 4 to 6 weeks.

Treatment of Fish

Disease, if allowed to progress unchecked, will bring about significant changes in the behavior pattern of fish (18). The migrating sockeye is particularly vulnerable to fungus (*Saprolegnia parasitica*) infection, especially if superficial wounds exist or if water temperatures are excessive, i.e. greater than 12° C (18). This fungus growth when left untreated progresses rapidly and in 2 to 3 weeks can not only kill the host but also infect other fish. The success of these studies depended upon finding a suitable treatment to prevent disease, particularly fungus. In preliminary experiments carried out at Courtenay, B.C., and the Vancouver Public Aquarium, various treatments were evaluated. Malachite green (18) was used at a concentration of 1:100,000 for 1 hour at 9° C, and the fish survived. However, the fungus continued to spread in spite of a second treatment 4 days later. Fish did not survive a 1.5-hour treatment at a concentration of 1:250,000 and a water temperature of 13 to 14° C on several occasions.

Salt baths, when given bi-weekly for a period of 3 hours at a level of 3% NaCl, were found to be very effective at water temperatures of 8–10° C in not only controlling the spread of fungus but also for the most part in preventing the appearance of the disease. This treatment was also effective at Courtenay where the water temperatures often fluctuated 5° C in a 12-hour period and reached an average daily high of 17.5–19.5° C for a period of several weeks. At Courtenay the treatment was given every 2 days. There were no deaths in a group of 50 sockeye between July 10 and August 17. If fish were wounded externally they often reacted violently to the salt bath and there was danger of further injury. To alleviate this risk the fish were first anaesthetized to a state

of mild sedation by the addition of 2-phenoxyethanol (reported to be antifungal (18)) to the aquarium water at a level of 20 ml/40 gallons and then treated with salt.

Testing Water Samples

The regular water supply entered the experimental aquarium at one end through rubber tubing, the outlet of which was placed one foot below the water surface. Air was supplied to the aquarium through a 3 ft × ¾ in. rubber hose fastened across the bottom of the tank. A series of ⅛-in. holes in the face of the hose gave a blanket of air which created a current across the top of the tank down the opposite end and across the bottom in a clockwise direction. Water samples to be tested for their effect on the fish were injected into the aquarium through a separate rubber hose connected at one end to a bilge pump seated in a large plastic bucket and then run parallel to the water line into the tank. The delivery rate of the test water varied between 2.8 and 3.0 gallons/minute.

Tests with potassium permanganate showed that the time interval for distribution of the compound throughout the whole of the tank was 2 minutes and 10 seconds from the time of injection. It required 6 hours and 20 minutes to clear all visible traces of permanganate from the tank. The entire segment of the room in which the experiments were performed was partitioned off with black plastic so that any movements of workers were invisible to the fish. The tank received illumination only from above. A peephole large enough to accommodate a camera lens was cut in the curtain at a point directly opposite the center of the glass side of the tank. The distance from the center of the glass to the peephole was 12 feet. A sheet of plywood which extended to the roof was mounted across the inlet end of the tank. The plywood supported the water lines and also permitted the operation of the various taps without disturbance of the fish. To encourage the fish to school at the end of the tank opposite to the test sample inlet, a shaded area was produced by placing black polyethylene over the extreme one-third of the tank opposite to the water inlet. A constant illumination of 150 ft-c in the center of the lighted area and 55 ft-c in the shaded area at the respective exposed water surfaces was provided by a single 500-watt lamp. The activity of the fish was recorded with a 16-mm Ciné camera.*

Exploratory studies showed that a minimum of 9 gallons of homestream water was required to produce a consistent response by the fish. A similar volume of Capilano River water evoked no response. The activity of the fish was watched for at least 15 minutes preceding, and 20 minutes following, the injection of the test water. Filming was begun 1 minute before, and continued for 7 minutes after, the start of the injection. The time required for injection was usually between 4 and 5 minutes. To obviate the possibility of contaminating the test waters with human repellents, plastic or rubber gloves were used whenever the test waters were handled (7, 8). The water samples were stored at 5° C in 4-gallon cans. The activity noted prior to the injection served as the

* Arriflex 16. Manufactured by Arnold and Richter, 89 Tuerkenstr., Munich 13.

control for the response obtained, if any, after the injection was started. If a response was obtained it usually occurred 1 or 2 minutes after the injection was completed. To ensure complete removal of test waters from the aquarium an interval of at least 24 hours was allowed between successive tests. On days when salt baths were given, the test was carried out preceding the salt treatment. Predominantly female fish were used and were changed frequently to avoid conditioned responses.

The water sample was said to elicit a positive response when (1) the school was dispersed, (2) the swimming speed of the fish increased, and (3) the fish did not reform the school for at least 3 minutes. Occasionally the fish broke the surface of the water. Where a positive response was obtained to a test water sample, the fish remained active for a period of from 10 to 40 minutes. This active period was sometimes punctuated by brief moments of schooling but not necessarily in the shaded area which the fish occupied during the control period.

The repellent used in this study consisted of about 2 liters of water in which two persons had washed their hands. The response in this test is quite different from that to the homestream water. In the response to the repellent, the fish sometimes show a little activity after the introduction of the repellent and then they move to the left-hand corner of the tank where they stay for several minutes. They school very closely and actually swim backwards into the corner. This behavior was observed only during the response to the repellent. Occasionally a fish moved a short distance from the school but returned very quickly.

Results

All the responses of the Great Central Lake and Cultus Lake sockeye to the different waters are recorded in Tables I and II and the sources of these samples are shown in Figs. 1 and 2.

TABLE I

Reaction of Great Central Lake fish to various waters*

	Great Central Lake	Lindsay Creek	Drinkwater Creek	Forest Camp Creek	Sweltzer Creek	Weaver Creek	Hand Rinse
Untreated water	++++ ++++	++	++	−−	−−−	−	+++
Lyophylized† volatile	+						
Lyophylized residue	−				−		
Ion-exchanged‡ water	+				−		
Autoclaved§	−						
Non-dialyzable‖ fraction	−						

*Each + or − represents one test. All test results are shown.
†Condensing flask held in dry ice – acetone bath; sample concentrated in water bath at 25° C to 30° C.
‡Resin column 30 cm in length and 3.5 cm in inside diameter. Ion exchange carried out at 5° C using Amberlite IR 120(H) and IRA 400 (OH).
§Steam heated at 100° C for 3 hours.
‖Dialyzed against running tap water (8–10° C) for 72 hours.

TABLE II

Reaction of Cultus Lake fish to various waters*

	Sweltzer Creek	Spring Creek	Frost Creek	C.L. Hatchery Creek	Weaver Creek	Steelhead Creek	Great Central Lake
Untreated water	+++	++	−−	++	−	−	−
Lyophylized† volatile	++						
Lyophylized residue	−						
Autoclaved‡	−						
Non-dialyzable§ fraction	−						

*Each + or − represents one test. All test results are shown.
†Condensing flask held in dry ice − acetone bath; sample concentrated in water bath at 25° C to 30° C.
‡Steam heated at 100° C for 3 hours.
§Dialyzed against running tap water (10° C) for 72 hours.

Great Central Lake fish responded positively to Great Central Lake water in eight separate tests (Table I). The water samples were taken at the outlet to the lake. The fish also responded positively to the water from Lindsay Creek and Drinkwater Creek, both of which feed a beach spawning area. There is also a small run of sockeye into Drinkwater Creek. The fish did not respond to the water from Forest Camp Creek, which to our knowledge does not support a sockeye population. Sweltzer and Weaver Creeks both have sockeye populations but are on the mainland and as would be anticipated the Great Central Lake fish gave no response to these waters. When Great Central Lake water was lyophilized at low temperature the fish responded to the volatile fraction but did not respond to the residue. When the same water was distilled under reduced pressure (water pump) with heating to about 70° C, the fish did not respond to either the distillate or to the residue. After Great Central Lake water was autoclaved at a 100° C for 3 hours the fish no longer responded to it.

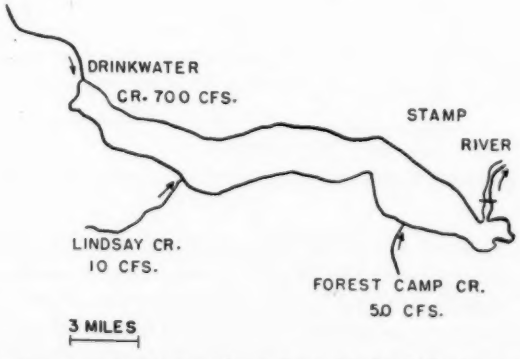

FIG. 1. Location of creeks feeding Great Central Lake, B.C., from which water was tested. Flow rates at time of adult sockeye migration.

Great Central Lake water was passed through a mixed bed of strong base and strong acid ion-exchange resins and the effluent produced a positive response. Great Central Lake water was dialyzed for 72 hours against running tap water at 8–10° C. The fish did not respond to the non-dialyzable material. Sweltzer Creek water was also passed through a mixed bed ion-exchange resin in order to determine if anything was eluted from the resin which may have been responsible for the positive response of Great Central Lake water treated in a similar fashion. The result was negative (Table I).

Cultus Lake fish responded positively to water from Sweltzer Creek through which they must pass in order to reach the Lake (Table II). They also responded positively to water from Spring Creek which feeds the main spawning area at the south end of the Lake (Fig. 2). Fish are also reported to spawn in

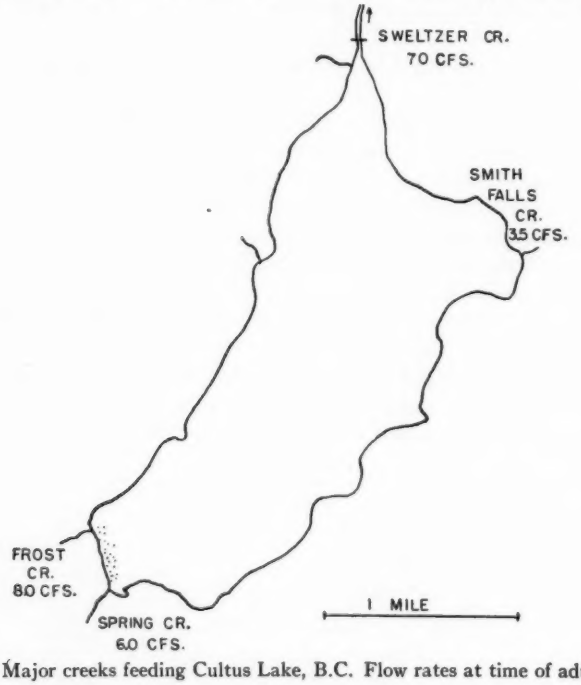

FIG. 2. Major creeks feeding Cultus Lake, B.C. Flow rates at time of adult sockeye migration.

the vicinity of Hatchery Creek and this water also elicited a positive response. The results on Frost Creek are of some interest, as this creek is very close to Spring Creek. However, there is no sockeye run into Frost Creek, whereas there is a small sockeye run into Spring Creek. The fish did not respond to the water from Frost Creek. Weaver and Steelhead Creeks are part of the Harrison River system, which is in close proximity to, but separate from, Cultus Lake.

Weaver Creek has a fairly large sockeye run, while Steelhead is barren. The Cultus Lake fish did not react to the water from either creek, nor did they react to the water from Great Central Lake. The responses of Cultus Lake fish to various fractions of Cultus Lake water confirmed the results obtained with corresponding fractions of Great Central Lake water tested on Great Central Lake fish, except that ion-exchanged water was not tested on Cultus Lake fish. Great Central Lake fish were repelled on three separate occasions by a rinse of human hands (Table I).

Many proposals have been made as to the type of clues by which salmon are guided to their spawning grounds. The effect of temperature (20) and CO_2 tension (21) in the selection of homestreams have been widely supported in the past, but with recent findings these proposals, for the most part, have been discarded (10, 15) in favor of chemical stimuli as the guiding factor in the selection of migratory routes at least during the fresh-water phase of the spawning migration.

It is acknowledged that the data presented here are based on a limited number of observations, particularly the fractionation studies. They are being presented at this time because the seasonal nature of the work will permit no further investigation until midsummer of 1961. With this in mind it is possible to draw certain tentative conclusions. The unknown active component (S) is water-soluble, neutral, dialyzable, and heat-labile in Great Central Lake water, while in Cultus Lake water it has been shown to be water-soluble, dialyzable, and heat-labile. Considering the high dilution of the samples, it is extremely unlikely that either CO_2 or water temperature would contribute to the responses obtained.

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TESTOSTERONE, CONJUGATED AND "FREE", IN THE BLOOD OF SPAWNED FRASER RIVER SOCKEYE SALMON (ONCORHYNCHUS NERKA)[1]

DOV GRAJCER AND D. R. IDLER

Abstract

Testosterone was isolated from both the "free" and conjugated steroid fractions obtained from the plasma of spawned female sockeye salmon. The structure of the steroid was confirmed by several criteria including a sulphuric acid chromogen and infrared spectra. Evidence is presented for the presence of testosterone in the conjugated steroid fraction prepared from spawned male sockeye. Several unsuccessful attempts were made to detect dehydroepiandrosterone and androsterone.

Introduction

Cortisol, cortisone, corticosterone, 17α-hydroxyprogesterone and its 20β-dihydroepimer, and 11-ketotestosterone have recently been isolated from the "free" steroid fraction obtained from the plasma of sockeye salmon (1, 2, 3). Reichstein's substance "S" has been tentatively established to be present while the evidence for aldosterone is inconclusive (1, 2, 4).

To our knowledge, no study has yet been reported concerning the isolation of conjugated steroids from fish blood. This investigation had its beginning in an attempt to establish the presence, in salmon plasma, of dehydroepiandrosterone and androsterone, the principal steroids in normal human plasma. These efforts led to the discovery of a conjugated steroid with a Δ^4-3-ketone moiety and this substance has been identified as testosterone.

Methods

Plasma

Blood was obtained from spawned Chilko Lake male and female sockeye salmon in September, 1959, spawned Cultus Lake females on November 28, 1960, and Siwash Bridge females on August 28, 1959. Fish were bled by severing the caudal artery and precautions were taken to exclude slime and excreta. The heparinized blood was chilled on ice, centrifuged promptly at 4000–5000 r.p.m., and the plasma stored in polyethylene containers on dry ice and later at −35° C. The "free" steroids were extracted from the alkalized plasma three times with 2.5 volumes of dichloromethane or ethyl acetate as previously described (3).

Isolation of Conjugated Steroids

Glucuronides.—The plasma residue from the extraction of the free steroids was treated with 2.5 volumes of ethanol to precipitate the proteins and the precipitate was removed by centrifugation at 9000 *g*. The precipitate was

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washed with 70% ethanol and the washings were added to the supernate. The entire procedure was carried out at 0° C. The supernate was evaporated *in vacuo* at ca. 30° C using a flash evaporator, the receiver of which was cooled in a dry ice – acetone mixture. The residue was suspended in a volume of water equivalent to that of the plasma and extracted three times with dichloromethane to remove any steroids which might have been released from the protein. Dichloromethane was used as a preservative. Versene (5 mg/100 ml) was added and the aqueous phase was adjusted to pH 4.5 and treated with β-glucuronidase (110 mg/100 ml),* at 37° C for 48 hours. The pH was then adjusted to 3.5 and the hydrolysis was continued for an additional 24 hours. Prior to use the β-glucuronidase was suspended in water and extracted three times with 2 volumes of dichloromethane. Steroids obtained by this procedure will be referred to as glucuronide conjugated ("G"). The steroids freed by the glucuronidase treatment were removed by three extractions with dichloromethane. The dichloromethane extract was washed three times with 0.1 volume of each of the following: 5% sodium bicarbonate, 0.1 *N* acetic acid, and water. The organic solvent was removed *in vacuo* as described above and the residue was partitioned between 70% methanol and hexane. The methanol was removed by flash evaporation, the residue taken up in water, and the "G" steroids extracted with dichloromethane.

Sulphate conjugates.—The aqueous phase from the extraction of the "G" steroids was made 2 *N* with respect to H_2SO_4 and 1 volume of ethyl acetate was added (5). The two phases were thoroughly equilibrated in a separatory funnel for several minutes and the ethyl acetate phase incubated at 37° C for 48 hours. The ethyl acetate was washed with sodium bicarbonate, 0.1 *N* acetic acid, and water. This fraction will be referred to as the sulphate conjugate (SO_4).

Residual conjugates.—The H_2SO_4 phase from the liberation of the SO_4 conjugates was neutralized with concentrated NaOH to a pH of 0.8 and the solution was continuously extracted with ether for 48 hours. A small amount of water was added to the ether (to prevent a high acid concentration when the volume of the solution was reduced) and the ether was removed at room temperature using a flash evaporator as described above. More water was added to the aqueous suspension of the residue which was then extracted with ethyl acetate and washed with sodium bicarbonate, acetic acid, and water. The substances released during this treatment will be referred to as the "pH 0.8" conjugates.

Column Chromatography

Neutral Woelm† activity 1 alumina was converted to activity 3 by shaking 15 g with 0.9 ml of water for 1 hour. A slurry of the adsorbent in benzene was poured into a 1-cm internal diameter column and the column of adsorbent was adjusted to ca. 20 cm by gently tapping the wall of the column. Gradient elution was carried out as previously described (6). The reservoirs contained

*Obtained from Worthington Biochemical Co., Freehold, New Jersey.
†Distributed by Alupharm Chemicals, P.O. Box 755, New Orleans, La.

4% ethanol in benzene, and benzene respectively. The column was operated at a flow rate of ca. 1.3 ml/minute. Eighty 10-ml fractions were collected, and then 4% ethanol in benzene was used to elute residual steroids.

Suitable aliquots of the residues representing the various steroid conjugate fractions were leached at room temperature with several small volumes of acetone and the acetone removed with a stream of nitrogen. The residue was dissolved in a small amount of benzene and applied to the adsorbent. A glass-wool plug was placed on top of the adsorbent in order to minimize plugging by insoluble material.

The solvent was removed from the individual or pooled fractions with the aid of a stream of nitrogen and a water bath maintained at 59° C. The 17-oxygenated steroids were determined by the Bongiovanni modification of the Zimmermann reaction. In this method N-benzyl trimethylammonium methoxide is substituted for KOH. The reaction was carried out at 4–8° C for 5 hours. The λ_{max} was 515 mμ and O.D. values were also read at 435 and 595 mμ, and Allan's correction was applied. When the Zimmermann reagent was used as a spray for paper chromatograms, one part of 40% N-benzyl trimethylammonium methoxide was mixed with 1 volume of ethanol and 2 volumes of a 2% ethanolic solution of metadinitrobenzene (7). The color was developed first at room temperature and then with heat. The method was sufficiently sensitive to detect 0.6 μg of dehydroepiandrosterone which had been run as a spot in the heptane – 80% methanol solvent pair.

Paper Chromatography

Paper was washed in a Soxhlet apparatus for long periods with acetic acid, ammonium hydroxide, and methanol as previously described (1). The paper was then washed with methanol for 48 hours by descending chromatography.

Hexane – propylene glycol (50% in MeOH) (HP-50), heptane:benzene (1:1) – 70% methanol (HM-70), toluene – 70% methanol (TM-70), and heptane – 80% methanol (HM-80) were used as solvent systems.

Solvents and Glassware

Dichloromethane was freshly distilled and stabilized with methanol. Methanol was added periodically during the removal of dichloromethane from steroid extracts. All other solvents were distilled. Glassware was washed with acid – chromic oxide cleaning solution followed by water, versene in aqueous methanol, and distilled water.

Sulphuric Acid Chromogens

Sulphuric acid (36 *N*) was added to the steroid to give a concentration of ca. 10 μg/ml. The spectra of the chromogens were determined after 2 hours, from 210–600 mμ, with the aid of a Beckman DK-1 spectrophotometer.

Infrared

Infrared spectra were recorded with a Beckman IR-4 double-beam spectrophotometer equipped with a beam-condensing system. Potassium bromide

(5–10 mg infrared grade) was spread over an area of ca. 0.5 inch^2 in a mortar warmed on a microheater to a temperature sufficient to volatilize dichloromethane. The steroid (ca. 20–80 μg) was taken up in 0.05–0.1 ml of dichloromethane and dispersed on the potassium bromide which was then gently mixed with a pestle. The pellet was pressed in the usual manner.

Experimental and Results

The glucuronide ("G") fraction from 650 ml of male plasma was chromatographed by gradient elution. Several appropriate standards (Table I) were treated in the same manner and quantitatively determined with the Zimmermann reagent. While it must be remembered that all Zimmermann-positive

TABLE I

Gradient elution of steroids from alumina*

Steroid	Tube No.
β-Etiocholanolone (50 μg)	10–13
Dehydroepiandrosterone (60 μg)	17–24
17β-OH-Androstan-3,11-dione (50 μg)	26–31
Isoandrosterone (100 μg)	26–32
Androsterone (70 μg)	33–38
Testosterone (200 μg)	34–39
Etiocholanolone (200 μg)	39–48
3β-OH-Androstan-11,17-dione (100 μg)	42–48

*See Method section of text for details.

steroids are not equally chromogenic, the results can be expressed as dehydroepiandrosterone equivalents, on which basis tubes 2–4 contained approximately 60 μg; 5–10, 35 μg; 14–15, 15 μg; 19, 10 μg; and 25–39, 100 μg. Tubes 2–4 of the 4% ethanol – benzene eluate contained a small amount of Zimmermann-positive material. In this paper we shall be primarily concerned with the material in tubes 25–39 which produced a Zimmermann chromogen reaching peak intensity (visual) in ca. 3 hours.

The "G" fraction from 860 ml of male plasma was chromatographed as above and the contents of tubes 25–39 were pooled and a sulphuric acid chromogen was run on one-half of the residue remaining after removal of the solvent. The chromogen had an absorption maximum at 308 mμ and a shoulder at 410 mμ. There were 60 μg of testosterone equivalents in these tubes calculated on the intensity of the absorption at 308 mμ. The remainder of the residue was applied to paper as a spot and chromatographed in the HM-80 solvent system for 3 hours. A spot, which absorbed ultraviolet light, was observed at 4.1–5.6 cm corresponding to testosterone and 17α-hydroxyprogesterone standards. The chromatogram was sprayed with the Zimmermann reagent but no chromogens developed until after the paper was heated. These experiments exhausted the supply of this fraction from male plasma.

The "G" fraction from 410 ml of plasma taken from Chilko Lake females was chromatographed using gradient elution. The residue from the contents of

tubes 25–39 was chromatographed in the HM-80 solvent system for 2.5 hours and an ultraviolet-absorbing material was present which had a mobility similar to the material obtained from male plasma. The substance was eluted from the paper and it had a λ_{max} in methanol at 238 mμ. The substance was rechromatographed in the same solvent system for 15 hours. The ultraviolet-absorbing material from plasma and testosterone both had $R_t = 0.93$ cm/hour, while the R_t values of 17α-hydroxyprogesterone and 11β-hydroxy-Δ^4-androsten-3,17-dione were 0.75 and 0.27 cm/hour, respectively. The plasma steroid was recovered from the paper, acetylated, and chromatographed for 2 hours in the HM-80 solvent system. The plasma substance and testosterone acetate both had $R_f = 0.79$. The reaction was essentially quantitative for both substances since no testosterone was detected after acetylation.

More plasma was processed and the steroid–acetate from a total of 525 ml was taken for the determination of the infrared spectrum (Fig. 1). The steroid–acetate recovered from the KBr pellet was equivalent to 2.5 μg/100 ml of plasma.

The steroid–acetate from plasma and testosterone acetate were hydrolyzed with methanolic NaOH and the steroids treated with chromic oxide. The steroids were recovered from the oxidation mixtures and chromatographed along with Δ^4-androsten-3,17-dione in the HM-80 solvent for 2 hours. All steroids had $R_f = 0.30$ and were detected both by their absorption of ultraviolet light and with the Zimmermann reagent.

Plasma (180 ml) obtained from Cultus Lake females was extracted with ethyl acetate and the "G" fraction obtained. The residue was chromatographed on paper for 24 hours using the HM-80 solvent system, and the region corresponding to testosterone eluted and rechromatographed in the same manner. Testosterone was determined quantitatively by ultraviolet absorption employing Allan's correction. The material from plasma exhibited maximum absorption at 238 mμ and gave O.D. readings at 220, 240, and 260 of 0.460, 0.870, and 0.291, respectively. There was 7.6 μg of testosterone released by β-glucuronidase from 100 ml of plasma based on the recovery of authentic testosterone through the procedure.

The "free" steroid fraction was prepared from 660 ml of plasma obtained from spawned Chilko Lake females. The residue was chromatographed in the HP-30 solvent system and the area of paper which would contain 17α-hydroxyprogesterone and testosterone was extracted with methanol. The methanol was removed, water added to the propylene glycol, and the steroids extracted with dichloromethane. The solvent was removed and the residue contained 90 μg of 17α-hydroxyprogesterone equivalents as determined by ultraviolet absorption employing Allan's correction. The residue was acetylated and chromatographed in the HBM-70 solvent system. There were two substances from the plasma strip which absorbed ultraviolet light. One substance ($R_f = 0.6$) had the mobility of 17α-hydroxyprogesterone and the other ($R_f = 0.88$) corresponded to testosterone acetate. Visual observation suggested that two

substances were present in comparable quantity. The infrared spectrum of the substance identified as testosterone is shown in Fig. 1.

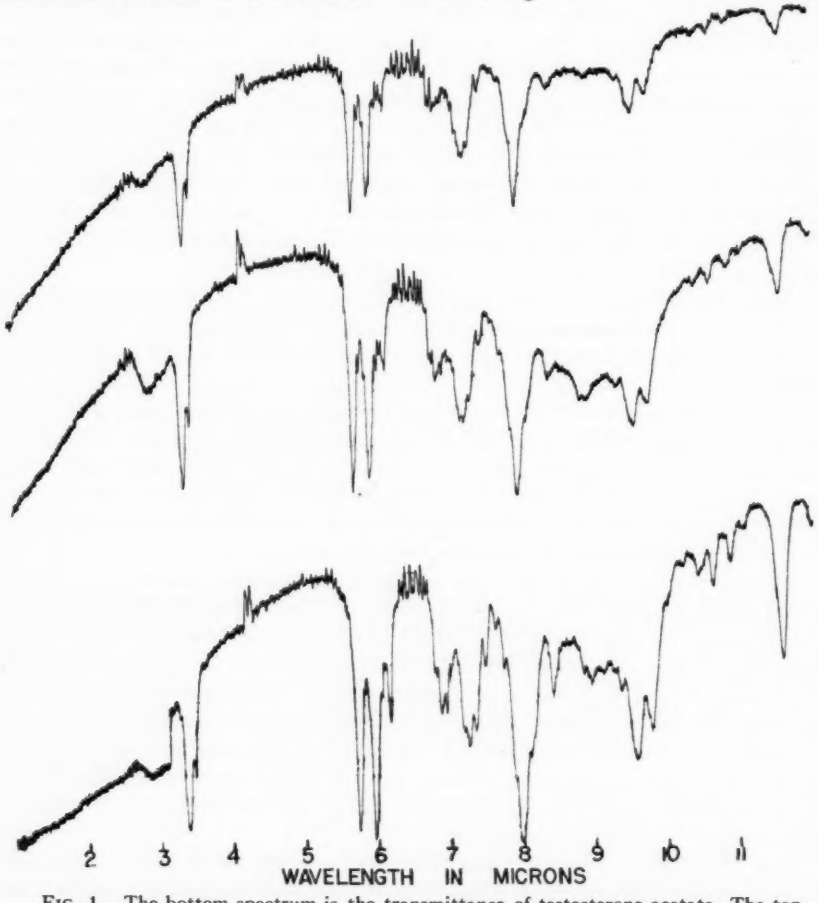

FIG. 1. The bottom spectrum is the transmittance of testosterone acetate. The top spectrum is of the acetate of the steroid obtained from the free steroid fraction, and the middle spectrum is of the acetate obtained from the conjugated fraction of plasma taken from spawned female sockeye salmon.

Several attempts were made to obtain evidence for the occurrence of dehydroepiandrosterone (DHA) or androsterone in salmon plasma. The "G" and "SO_4" fractions from 218 ml of plasma from spawned Chilko Lake females were chromatographed as streaks in the HP-50 solvent system. The areas of the paper corresponding to DHA and androsterone were eluted and the residues chromatographed as spots. No Zimmermann-positive material was detected. The "G" fraction from 136 ml of plasma taken from spawned Chilko Lake

males was treated in a similar manner and again no material with the mobility of either DHA or androsterone was detected. The "SO_4" fractions from 544 and 136 ml of the same plasma also contained no detectable DHA or androsterone. The "pH 0.8" fraction from 136 ml and the "SO_4" fraction from 47 ml of the same plasma also showed no evidence of DHA or androsterone. The "G" fraction of the plasma from 412 ml of spawned Chilko Lake females was chromatographed by gradient elution. The contents of the tubes, which should have contained DHA and androsterone had they been present, were chromatographed in the HM-80 solvent system and no ultraviolet-absorbing or Zimmermann-positive substances were detected. The "SO_4" conjugate from the plasma (870 ml) of spawned Chilko Lake females was chromatographed on a 15-cm strip of paper in the HP-50 solvent system. The Zimmermann reaction was carried out on 10% of the eluate from paper strips taken from the areas which would be occupied by DHA or androsterone and no detectable chromogen was produced. The remainder of the material with the mobility of androsterone and 25% and 10% respectively of the material corresponding in chromatographic mobility with DHA and testosterone were rechromatographed as spots in the same solvent system and no Zimmermann-positive material was detected.

The "G" and "SO_4" fractions from 224 ml and 560 ml of plasma, respectively, taken from females captured at Siwash Bridge were chromatographed by gradient elution. The Zimmermann reaction was carried out on the contents of tubes 1–80 and only tube 2 contained a significant amount of chromogenic material. Another aliquot equivalent to 47 ml of plasma was chromatographed in HP-50 alongside 17α-hydroxyprogesterone dehydroepiandrosterone, androsterone, and etiocholanolone. There was a faint Zimmermann-positive spot with the mobility of 17α-hydroxyprogesterone (or testosterone) and nothing corresponding to the other reference substances. The "pH 0.8" conjugates prepared from 170 ml of plasma taken from spawned Chilko Lake males were subjected to column chromatography. Only a trace of Zimmermann-positive material was detected.

Discussion

The failure, in the present study, to detect either DHA or androsterone in any of several plasma samples is of some interest since both these steroids are present in relatively high concentration in human peripheral plasma. Dehydroepiandrosterone has been isolated and positively identified in human plasma where it is reported to occur at a concentration of 57.5 ± 10.5 μg/100 ml (8). Androsterone has been reported to occur at a concentration of 18 μg and 22.5 μg/100 ml, respectively, in the plasma of normal females and males (9).

The first demonstration of the conjugation of a steroid at a position other than the C(3) hydroxyl group was the isolation of testosterone-17β-glucuronide from the urine of a patient receiving large doses of testosterone propionate (10). Testosterone is converted in low yield by rat liver slices in vitro to a substance positively identified as testosterone-17β-glucuronide (11). Unconjugated testo-

sterone has recently been isolated from human systemic blood following the administration of human chorionic gonadotrophin (12). To our knowledge conjugated testosterone has not been previously isolated from blood.

There was 7.6 μg/100 ml of testosterone in the "G" fraction of plasma taken from spawned Cultus Lake females. In another experiment 2.5 μg/100 ml of testosterone was recovered from 525 ml of plasma taken from spawned Chilko Lake females. This substance had been taken through two paper chromatograms, one column chromatogram, acetylation, and recovery from a KBr pellet. The recovery from each paper chromatogram can be estimated from the recovery of pure steroids to be ca. 85%. Since no testosterone was detected on the paper chromatogram following acetylation the recovery was ca. 95%. The recovery from the KBr pellet and column chromatogram can only be estimated approximately, ca. 85% each. There were therefore ca. 5.1 μg of testosterone released from each 100 ml of plasma by β-glucuronidase. This estimated level of testosterone in the spawned Chilko Lake females was therefore comparable to the level accurately determined in the "G" fraction of plasma obtained from Cultus Lake spawned females.

The plasma sample obtained from females captured at Siwash Bridge, approximately 3 weeks prior to spawning, contained no detectable Zimmermann-positive substance in tubes 4–80 following column chromatography. It must be remembered that the Zimmermann reaction as used in this study is quite ineffective on 17-hydroxysteroids and the intensity of the color produced with 3-ketosteroids reaches a maximum in 3 hours and has decreased in intensity by the time the tubes are read at the end of 5 hours, which is the optimum time for 17-ketosteroids. Since significant amounts of steroids with a Δ^4-3-ketone structure were not expected to occur as conjugates at the time these chromatograms were run testosterone, unless present at a concentration of ca. 2 μg/100 ml, would probably not have been detected. However, testosterone should have been detected by paper chromatography if it had been present in significant quantities. It has been shown in a separate investigation that this plasma sample contained only 3.8 μg/100 ml of "free" 17α-hydroxyprogesterone plus testosterone, while the plasma of spawned Cultus Lake females, which contained 7.6 μg of testosterone in the "G" fraction, contained 7.7 μg/100 ml of testosterone and 9.8 μg/100 ml of 17α-hydroxyprogesterone in the "free" steroid fraction. The steroids were determined following paper chromatography in the heptane – 80% methanol solvent system for 16 hours (13). It can be concluded that testosterone was present at a considerably higher concentration in both the "G" and "free" fractions of spawned females than in the corresponding fractions prepared from the plasma of females captured prior to arrival in the spawning area. Testosterone in the combined "free" and "G" fractions of plasma from spawned Chilko Lake females was 15.3 μg/100 ml (7.7 μg plus 7.6 μg). By contrast the level of "free" testosterone in normal human peripheral plasma of both sexes is extremely small and values ranging from 0.1 μg to 0.4 μg/100 ml have recently been reported (14). It can also be

concluded that testosterone is present at a considerably higher concentration in both the "G" and "free" fractions of spawned females than in the corresponding fractions prepared from the plasma of females captured prior to arrival on the spawning grounds.

17α-Hydroxyprogesterone was previously isolated from the "free" steroid fraction of plasma taken from females en route to Adams River (1). Testosterone and 17α-hydroxyprogesterone were both isolated from the "free" steroid fraction of plasma taken from spawned Chilko Lake females in the present study. Testosterone and 17α-hydroxyprogesterone are difficult to separate by paper chromatography although the HM-80 solvent system will effect an adequate separation if run long enough (i.e., 16 hours). The fact that testosterone acetylates under very mild conditions, while 17α-hydroxyprogesterone does not, forms the basis for the separation used in this study. The distribution of the two "free" steroids in plasma samples taken from fish at various stages of sexual maturity has been studied and the data will be included in a separate submission (13).

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TRANSFORMATION DE L'ACIDE γ-HYDROXYGLUTAMIQUE EN ALANINE ET EN ACIDE GLYOXYLIQUE[1]

L. P. Bouthillier, Y. Binette et G. Pouliot

Abstract

A method is described for the synthesis of γ-hydroxyglutamic-5-C^{14} acid (racemic mixture). Doses of this substance were administered intraperitoneally to rats and among the amino acids isolated from the tissue proteins, glycine showed the highest radioactivity. This finding is compatible with the postulated theory that γ-hydroxyglutamic acid may be cleaved with the formation of alanine and glyoxylic acid, the latter being normally converted into glycine by amination in the animal tissues. Following the incubation of γ-hydroxyglutamic-5-C^{14} acid and also γ-hydroxyglutamic-2-C^{14} acid in the presence of rat liver homogenates, evidence was obtained, by the carrier technique, for the direct formation of radioactive glyoxylic acid and alanine; no trace of glutamic acid or aspartic acid could be detected in the incubation media. As a result of a comparative study of the breakdown in vitro of the racemates of γ-hydroxyglutamic-5-C^{14} acid, it was found that the isomers of erythro-DL-γ-hydroxyglutamic acid are utilized to a much greater extent than the isomers of threo-DL-γ-hydroxyglutamic acid.

Introduction

Les travaux récents de Adams et Goldstone (1) démontrent bien que l'acide L-γ-hydroxyglutamique est un produit du catabolisme de la L-hydroxyproline. Les premières recherches de Gianetto et Bouthillier (2) sur le métabolisme de la DL-hydroxyproline-2-C^{14}, suivies plus tard de celles de Wolf et ses collaborateurs (3), soulignaient déjà le rôle de l'acide γ-hydroxyglutamique dans le métabolisme de la L-hydroxyproline. Benoiton et Bouthillier (4) ont les premiers synthétisé de l'acide γ-hydroxyglutamique (mélange des deux racémiques) et ils ont fait chez le rat une étude préliminaire du métabolisme de cet acide aminé marqué au radiocarbone en position 2 (5). Des travaux ultérieurs (6) ont montré que l'acide γ-hydroxyglutamique-2-C^{14} injecté à des rats contribuait à la formation d'alanine, d'acide glutamique et d'acide aspartique isotopiques, dont les valeurs relatives de radioactivité étaient respectivement 1.5, 1.2 et 1.0. Peu de temps auparavant, Wolf (3) et ses collaborateurs avaient obtenu des résultats semblables, après avoir administré à des rats de la DL-hydroxyproline-2-C^{14}. Pour expliquer la formation d'alanine radioactive, ces auteurs ont formulé l'hypothèse d'une scission moléculaire de l'acide γ-hydroxyglutamique entre les atomes de carbone 3 et 4, donnant ainsi naissance à de l'alanine et à de l'acide glyoxylique.

Nous avons donc entrepris des recherches dans le but de vérifier cette hypothèse, en étudiant dans les tissus du rat, in vivo et in vitro, le métabolisme de l'acide γ-hydroxyglutamique marqué au radiocarbone en position 5 ou en position 2 de la molécule. Les travaux que nous décrivons dans le présent

[1]Manuscrit reçu le 22 juin 1961.
Contribution du Département de Biochimie, Faculté de Médicine, Université de Montréal, Casier postal 6128, Montréal, Qué.

article ont fait, en grande partie, l'objet de communications antérieures (7, 8). Les faits que nous rapportons militent en faveur de l'hypothèse de Wolf, puisqu'ils démontrent que chez le rat, du moins dans le tissu hépatique, l'acide γ-hydroxyglutamique donne lieu directement à la formation d'alanine et d'acide glyoxylique. Dekker (9) a montré plus tard qu'un extrait enzymatique de foie de rat catalyse cette réaction. Il est intéressant de mentionner aussi que des auteurs japonais (10) ont isolé du foie de rat un enzyme qui catalyse réversiblement la réaction:

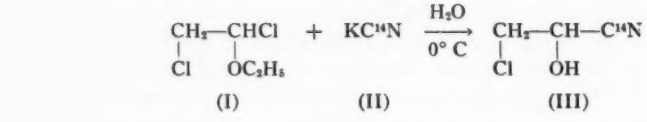
acide pyruvique + acide glyoxylique ⇄ acide γ-hydroxy-α-cétoglutarique.

Dans le présent article, nous décrivons la méthode que nous avons imaginée et mise au point pour la synthèse de l'acide γ-hydroxyglutamique-5-C^{14} (mélange des deux racémiques).

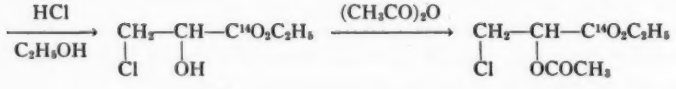
Partie expérimentale

(*A*) *Synthèse de l'acide γ-hydroxyglutamique-5-C^{14}*

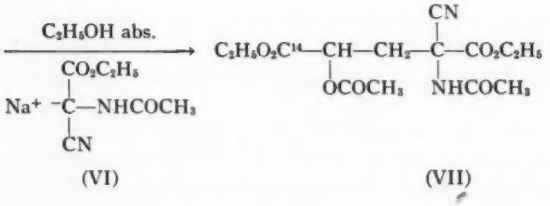
Le procédé que nous avons employé pour préparer ce composé radioactif se trouve illustré et décrit ci-dessous. Il est à remarquer que la dernière phase de ce procédé est en tous points celle de la méthode qui fut décrite par Benoiton et Bouthillier (5).



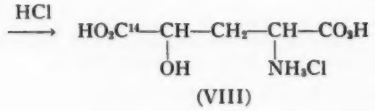
(I) (II) (III)



(IV) (V)



(VI) (VII)



(VIII)

1. Préparation de l'hydroxy-2 chloro-3 propionitrile-1-C¹⁴ (III)

Selon la technique employée par Houben et Pfankuck (11), on prépare une solution de 33 mg de cyanure de potassium radioactif (1 mc) et de 1 g de cyanure de potassium normal dans 2 ml d'eau. À cette solution préalablement refroidie à 0° C et maintenue en agitation, on ajoute goutte à goutte 3 g de dichloro-1,2 éthoxyéthane; on abandonne ½-heure dans un bain d'eau et de glace. Au bout de ce temps, on ajoute 3 ml d'eau et on acidifie légèrement avec de l'acide chlorhydrique. On extrait à l'éther, on déshydrate la solution et on évapore ensuite l'éther. On distille le résidu sous vide et on recueille la fraction qui passe entre 70 et 120° C, sous la pression de 12 mm de mercure. Nous avons ainsi obtenu 1.38 g de produit III presque pur, soit un rendement voisin de 85%, calculé d'après la quantité de cyanure de potassium employée.

2. Préparation de l'hydroxy-2 chloro-3 propionate-1-C¹⁴ d'éthyle (IV)

Selon le procédé décrit par Matheson et Blaikil (12), on dissout dans 2 g d'éthanol absolu 1.38 g d'hydroxy-2 chloro-3 propionitrile-1-C¹⁴ (III) et on ajoute 0.3 g d'acide chlorhydrique concentré. Dans cette solution maintenue à 30–35° C, on fait barbotter du gaz chlorhydrique desséché, jusqu'à précipitation de cristaux de chlorure d'ammonium. On chauffe ensuite pendant 4 heures dans un bain d'eau à 85° C. On sépare par centrifugation les cristaux de chlorure d'ammonium formé et on les lave une fois avec de l'éthanol. On mélange la liqueur alcoolique surnageante avec l'alcool de lavage et on évapore l'alcool sous pression réduite. Par distillation fractionnée du résidu, on recueille la fraction qui passe entre 102 et 104° C sous la pression de 25 mm de mercure.

Nous avons obtenu 1.37 g du dérivé ester (IV) dont le point de fusion non corrigé était de 35–37° C.

3. Préparation de l'acétoxy-2 chloro-3 propionate-1-C¹⁴ d'éthyle (V)

On effectue l'acétylation du produit IV selon la méthode de Kenyon *et al.* (13). Dans 3 ml d'anhydride acétique, on dissout 1.37 g d'hydroxy-2 chloro-3 propionate-1-C¹⁴ d'éthyle et on chauffe durant 20 heures dans un bain d'eau bouillante. On évapore l'anhydride acétique contenu en excès de même que l'acide acétique formé et on distille le résidu sous faible pression. Le dérivé acétylé distille vers 100–103° C sous la pression de 15 mm de mercure.

Nous avons obtenu 1.5 g d'acétoxy-2 chloro-3 propionate-1-C¹⁴ d'éthyle (V), soit un rendement de 89%.

4. Obtention d'acide γ-hydroxyglutamique-5-C¹⁴

Selon la méthode préconisée par Benoiton et Bouthillier (4), nous avons fait la condensation d'acétamidocyanoacétate d'éthyle (VI) avec de l'acétoxy-2 chloro-3 propionate-1-C¹⁴ d'éthyle (V). Nous avons fait l'hydrolyse du produit de condensation (VII) en milieu chlorhydrique à 20% et nous avons ainsi obtenu le chlorhydrate de l'acide γ-hydroxyglutamique-5-C¹⁴ (VIII). Nous avons purifié ce composé par chromatographie sur une colonne (2.5×45 cm) de résine Dowex 1, en employant de l'acide acétique 1 *N* comme agent d'élution. Nous avons récolté finalement 420 mg d'acide γ-hydroxyglutamique-5-C¹⁴

monohydraté (mélange des deux racémiques), soit un rendement global d'environ 15% calculé sur la base du cyanure employé. La radioactivité du produit fut évaluée à 7.5×10^4 coups/minute/mg. Le taux d'impureté radioactive, tel qu'établi par radiochromatographie sur papier, ne dépassait pas 2%. De plus, nous avons caractérisé notre produit de synthèse par chromatographie sur papier dans différents solvants, par comparaison avec des échantillons authentiques d'acide γ-hydroxyglutamique normal et aussi par oxydation en acide aspartique (4).

(*B*) *Protocole des expériences biologiques*

1. Expériences in vivo

Trois rats mâles de souche Wistar ont reçu chacun, par injection intrapéritonéale, 1 ml d'une solution aqueuse (pH 7.3) de 25 mg d'acide γ-hydroxyglutamique-5-C^{14}. Les animaux furent sacrifiés 90 minutes plus tard et nous avons prélevé de chaque animal: le foie, les poumons, le coeur, la rate, les reins, les testicules, le cerveau, le tissu intestinal (libre de matières fécales) et le plus possible de tissus musculaires et de tendons, soit environ 25 g de tissus frais.

Nous avons déchiqueté l'ensemble des tissus de chaque animal dans un broyeur (Waring Blendor), en présence d'acide trichloracétique à 20%. Nous avons séparé par centrifugation les matières protéiques coagulées; nous les avons ensuite asséchées et délipidées par traitement à l'acétone et à l'éther. Nous avons fait l'hydrolyse de la poudre de protéines brutes dans de l'acide chlorhydrique 6 *N*, par chauffage à reflux durant 20 heures et nous avons extrait à l'éther les matières grasses libres que contenait l'hydrolysat. Par chromatographie sur une colonne (4.5 × 100 cm) de résine Dowex 50 × 4 (200–400 mailles/pouce), nous avons isolé de l'hydrolysat, par l'emploi d'acide chlorhydrique 1 *N* comme agent d'élution, un certain nombre d'acides aminés dont la sérine, l'alanine et la glycine. Comme celle-ci se trouvait quelque peu souillée d'acide glutamique, nous avons séparé ces deux acides aminés à l'aide de résine Dowex 1 × 4 et d'acide acétique 1 *N* comme agent d'élution.

Nous avons oxydé (14) complètement des échantillons des acides aminés purifiés et nous avons recueilli dans chaque cas la totalité de l'anhydride carbonique sous forme de carbonate de baryum, dont nous avons mesuré la radioactivité. Nous avons calculé la radioactivité totale qui existait sous forme de chacun des acides aminés, en exprimant celle-ci en coups par minute. Nous avons ensuite calculé les rapports des valeurs de radioactivité, en comparant chaque valeur à celle de l'acide aminé le moins radioactif du groupe.

2. Expériences in vitro

Nous avons fait un certain nombre d'incubations à la température de 38° C, pour une durée de 2 heures. Chaque préparation réunissait 5 mg d'acide γ-hydroxyglutamique radioactif (5-C^{14} ou 2-C^{14}), 5 mg de glyoxylate de sodium ou 5 mg d'alanine non radioactifs comme agents de dilution et 6 ml d'homogénat de foie de rat. Selon la technique usuelle, nous avons préparé les homogénats en pulvérisant chaque fois 1 g de tissu frais en présence d'un mélange refroidi

de 3 ml de Na_2HPO_4 0.04 *M* et de 2 ml de $MgSO_4$ 0.04 *M*. Au terme de l'incubation, nous avons fait coaguler les matières protéiques par addition de 10 ml d'éthanol et nous avons chauffé quelques minutes à la température d'ébullition. Après élimination du coagulum par centrifugation, nous avons préparé des radiochromatogrammes sur papier de portions aliquotes du liquide surnageant. Comme solvants chromatographiques, nous avons employé le mélange phénol–eau (4:1) et aussi le mélange butanol–pyridine–eau (5:5:2).

Résultats et Discussion

Au Tableau I se trouvent réunies les valeurs de la radioactivité de la glycine, de la sérine et de l'alanine constitutives des protéines tissulaires des rats qui ont reçu en injection intrapéritonéale de l'acide γ-hydroxyglutamique-5-C^{14}. Nous

TABLEAU I

Radioactivité comparée de la glycine, de la sérine et de l'alanine constitutives des protéines tissulaires des rats qui ont reçu en injection intrapéritonéale de l'acide γ-hydroxyglutamique-5-C^{14}

Acides aminés	Radioactivité totale, coups/minute				Rapports des valeurs de radioactivité			
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 1	Rat 2	Rat 3	Rat 4
Glycine	38,785	48,785	26,460	41,265	9.5	10.7	12.2	12.5
Sérine	21,410	41,455	17,910	34,735	5.3	9.1	8.3	10.5
Alanine	4,060	4,550	2,160	3,300	1.0	1.0	1.0	1.0

constatons que la glycine contenait beaucoup plus de radiocarbone que les deux autres acides aminés. Ce phénomène s'explique très bien en admettant qu'il y ait eu d'abord scission de la molécule d'acide γ-hydroxyglutamique-5-C^{14}, au point de liaison des atomes de carbone 3 et 4, avec production d'acide glyoxylique-1-C^{14}. Il est tout naturel qu'une certaine quantité de ce composé ait participé à une réaction de transamination pour former de la glycine-1-C^{14}. Que de la glycine radioactive se soit ensuite transformée en sérine-1-C^{14}, cela n'a rien d'étonnant puisque cette réaction a lieu normalement chez le rat (15, 16). Les données consignées au Tableau II n'indiquent-elles pas d'ailleurs que

TABLEAU II

Radioactivité du groupement carboxylique de la glycine et de la sérine exprimée en pourcentage de la radioactivité totale de leur molécule

Acides aminés	Rat 1	Rat 2	Rat 3	Rat 4
Glycine	90	93	91	93
Sérine	90	97	95	94

la presque totalité du radiocarbone que contenaient la glycine et la sérine tissulaires se trouvait localisée dans leur groupement carboxylique. Ces chiffres furent obtenus en comparant les valeurs de radioactivité totale d'échantillons

de glycine et de sérine à celles de l'anhydride carbonique libéré de ces acides aminés par l'action de la ninhydrine. Voilà donc des faits qui militent déjà fortement en faveur de la théorie de Wolf pour ce qui a trait à la formation d'acide glyoxylique aux dépens de l'acide γ-hydroxyglutamique.

Pour ce qui est maintenant de la radioactivité de l'alanine (Tableau I), il est permis de supposer que la sérine-1-C^{14} en a été la source. Il est d'ailleurs parfaitement établi que la sérine, sous l'action de la sérine déshydrase, conduit à l'acide pyruvique lequel, on le sait, engendre de l'alanine par le processus de la transamination.

Nous en arrivons maintenant à parler des expériences que nous avons faites in vitro. Nous avons tenté de démontrer que l'acide γ-hydroxyglutamique produit directement, par désaldolisation, de l'acide glyoxylique et de l'alanine.

Dans une première série d'expériences, nous avons préparé deux milieux d'incubation; chacun réunissait un homogénat de foie de rat, de l'acide γ-hydroxyglutamique-5-C^{14} et enfin du glyoxylate de sodium comme agent de dilution (voir Protocole des expériences biologiques). Au terme de chaque incubation, nous avons précipité les protéines par de l'éthanol à chaud et nous avons ajouté au filtrat 35 mg de glyoxylate de sodium et environ 100 mg de 2,4-dinitrophénylhydrazine en solution dans de l'acide chlorhydrique 2 *N*. Nous avons fait l'extraction à l'éther de la 2,4-dinitrophénylhydrazone formée; nous l'avons ensuite extraite de la liqueur éthérée à l'aide d'une solution aqueuse faible de carbonate de sodium, que nous avons ensuite acidifiée par l'acide chlorhydrique. Nous avons fait l'extraction de l'hydrazone par l'acétate d'éthyle et l'évaporation de ce solvant a fourni des cristaux de 2,4-dinitrophénylhydrazone de l'acide glyoxylique. Par recristallisation de ce produit dans de l'éthanol à 50% nous avons obtenu des cristaux qui fondaient à 188° C, chiffre donné par les auteurs (17). Des radiochromatogrammes nous ont permis de vérifier le degré de pureté de cette substance, puisque ceux-ci ne présentaient qu'une seule zone radioactive. Nous avons mesuré la radioactivité d'échantillons de l'hydrazone et nous avons calculé une radioactivité totale de 4200 coups/minute dans un cas et environ 5000 coups/minute dans l'autre. Considérant le haut degré de pureté de l'hydrazone et sa radioactivité hautement significative, il nous est permis d'affirmer que, dans les conditions de nos expériences, il s'est formé directement de l'acide glyoxylique radioactif aux dépens de l'acide γ-hydroxyglutamique-5-C^{14}.

Dans une seconde série d'expériences, nous avons fait l'incubation (voir Protocole des expériences biologiques) de l'acide γ-hydroxyglutamique-2-C^{14} (mélange des deux racémiques) (5) en présence d'un homogénat brut de foie de rat, dans lequel nous avions ajouté de la L-alanine normale. Après déprotéinisation du milieu d'incubation, nous avons fait la chromatographie du filtrat sur une colonne (2.5×45 cm) de résine Dowex 50×4 (200–400 mailles/pouce) en employant de l'acide chlorhydrique 1 *N* comme agent d'élution. Dans deux expériences distinctes, l'alanine que contenaient les fractions (15 ml) n° 41–47 de l'éluat accusait une radioactivité totale de 38,000 et de 50,000 coups/minute;

ces valeurs représentaient environ 35 et 46% de la radioactivité ajoutée dans chaque milieu d'incubation sous forme d'acide γ-hydroxyglutamique-2-C^{14}. À part l'acide γ-hydroxyglutamique-2-C^{14} résiduel, l'alanine était la seule autre substance radioactive d'importance que contenait chaque milieu d'incubation; le degré de pureté de l'alanine fut d'ailleurs confirmé par radiochromatographie sur papier dans les solvants phénol–eau et butanol–pyridine–eau. Il ne fait donc aucun doute que, dans le milieu d'incubation, de l'alanine radioactive a pris naissance directement aux dépens de l'acide γ-hydroxyglutamique-2-C^{14}. Les résultats de ces expériences (8) concordent d'ailleurs avec ceux qu'a rapportés Dekker (9).

Nous tenons à souligner que nous n'avons trouvé dans les milieux d'incubation aucune trace d'acide aspartique ou d'acide glutamique radioactifs. Voilà un fait tout aussi intéressant qu'imprévu, puisque les résultats des expériences faites chez le rat avec de la DL-hydroxyproline-2-C^{14} (2, 3) et aussi avec le mélange des deux racémiques de l'acide γ-hydroxyglutamique-2-C^{14} (5) avaient laissé croire à l'existence de deux processus de transformation de l'acide γ-hydroxyglutamique, l'un conduisant à l'acide aspartique, l'autre à l'acide glutamique. Ne pourrait-on pas d'ailleurs expliquer les résultats de ces expériences antérieures faites in vivo, en admettant que de l'alanine-2-C^{14}, dont la formation ne fait pas de doute, a transmis une partie de son radiocarbone à l'acide aspartique et à l'acide glutamique, grâce à la participation active des céto-acides de ces trois acides aminés dans les réactions du cycle des acides tricarboxyliques.

Au sujet de l'absence de formation d'acide aspartique, par voie directe, aux dépens de l'acide γ-hydroxyglutamique, qu'il nous suffise seulement de signaler ici les résultats d'expériences que nous avons faites chez le rat avec de l'acide γ-hydroxyglutamique-4-C^{14} (8). Cette fois, l'alanine, l'acide aspartique et l'acide glutamique isolés des protéines tissulaires étaient tout aussi radioactifs les uns que les autres. Si vraiment une certaine quantité d'acide γ-hydroxyglutamique-4-C^{14} s'était transformée en acide aspartique-4-C^{14}, par un processus particulier, nous aurions sans doute retrouvé une forte proportion de la radioactivité totale de l'acide aspartique dans l'anhydride carbonique de décarboxylation. Or tel ne fut pas le cas; tout au contraire, moins de 10% de la radioactivité totale de l'acide aspartique tissulaire se trouvait localisé dans les deux groupements carboxyliques à la fois. Dans une prochaine publication, nous ferons connaître les résultats détaillés de ces expériences.

L'acide γ-hydroxyglutamique-5-C^{14} que nous avons employé dans nos expériences était un mélange des racémiques érythro et thréo. Il devenait intéressant de savoir si les deux racémiques ne seraient pas décomposés à des taux différents, par incubation de chacun en présence d'un homogénat de tissu hépatique du rat. Nous avons donc procédé à la séparation des deux racémiques selon la méthode fort simple préconisée par Benoiton et ses collaborateurs (18), en opérant avec 100 mg d'acide γ-hydroxyglutamique-5-C^{14} de synthèse. Nous avons recueilli 56 mg de cristaux du chlorhydrate de la lactone de l'acide thréo-

DL-γ-hydroxyglutamique-5-C^{14}. Par évaporation à sec de la liqueur mère, en présence d'azote pour éviter toute décomposition de l'acide aminé, nous avons obtenu des cristaux du chlorhydrate de l'acide érythro-DL-γ-hydroxyglutamique-5-C^{14}. Bien que celui-ci se trouvait quelque peu souillé du racémique thréo, il était tout de même intéressant de faire une étude comparée de la dégradation biologique des deux racémiques, en opérant dans les mêmes conditions d'incubation déjà décrites. Notons qu'au pH 7.4, l'acide thréo-DL-γ-hydroxyglutamique n'existait donc plus sous forme lactonisée puisque les deux racémiques résiduels, une fois isolés des milieux d'incubation, présentaient les mêmes valeurs de R_f en chromatographie sur papier.

Nous avons préparé des chromatogrammes sur papier de portions aliquotes des milieux d'incubation, après que ceux-ci eurent été déprotéinisés à la façon usuelle. Nous avons établi ensuite la distribution de la radioactivité sur chaque chromatogramme développé dans le mélange phénol–eau (4:1) et nous avons repéré deux zones fortement radioactives et bien distinctes: l'une était attribuable à la glycine et l'autre à l'acide γ-hydroxyglutamique résiduel. Au Tableau III figurent les valeurs relatives de la radioactivité de la glycine et de

TABLEAU III

Résultats de l'étude comparative in vitro de la dégradation des racémiques thréo et érythro de l'acide γ-hydroxyglutamique-5-C^{14}
(Valeurs relatives de la radioactivité de l'acide γ-hydroxyglutamique résiduel et de la glycine, exprimées en pourcentage de la radioactivité totale mesurée sur chaque chromatogramme)

Expérience 1		Expérience 2	
Incubation d'acide thréo-DL-γ-hydroxyglutamique-5-C^{14}		Incubation d'acide érythro-DL-γ-hydroxyglutamique-5-C^{14}	
Substrat résiduel	66	Substrat résiduel	24
Glycine	30	Glycine	73
Autres	4	Autres	3

l'acide γ-hydroxyglutamique résiduel, celles-ci étant exprimées en pourcentage de la radioactivité totale mesurée sur chaque chromatogramme. À l'examen de ces valeurs, il ressort que le taux de dégradation, par désaldolisation, de l'acide érythro-DL-γ-hydroxyglutamique-5-C^{14} fut nettement supérieur à celui de l'acide thréo-DL-γ-hydroxyglutamique-5-C^{14}. Notons ici que l'hydroxyproline naturelle et l'acide érythro-L-γ-hydroxyglutamique s'apparentent en raison de la configuration de leurs deux atomes de carbone asymétrique. À l'examen des résultats de l'expérience 2, il appert que les deux isomères optiques de l'acide érythro-DL-γ-hydroxyglutamique-5-C^{14} et non pas un seul ont été en bonne partie dégradés. En effet, en supposant que l'isomère L aurait été complètement décomposé, ce qui est peu probable, nous déduisons que même dans ce cas près de la moitié de l'isomère D aurait subi la dégradation. Pour ce qui est maintenant du taux relatif de décomposition de chaque isomère de l'acide thréo-γ-hydroxyglutamique-5-C^{14} dans l'expérience 1, il nous est im-

possible pour l'instant de faire la part de chacun. Ce ne sont là que des résultats préliminaires et nos recherches se continuent.

Remerciements

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FATE OF PHENYLALANINE-C^{14} AND CINNAMIC ACID-C^{14} IN *MALUS* IN RELATION TO PHLORIDZIN SYNTHESIS[1]

P. N. AVADHANI[2] AND G. H. N. TOWERS

Abstract

Both phenylalanine-C^{14} and cinnamic acid-C^{14} were utilized in the synthesis of phloridzin-C^{14} by *Malus* leaf disks, although this synthesis could be demonstrated only with young leaves. Two compounds, tentatively identified as a cinnamyl-glucose ester and a *p*-coumaryl-glucose ester, were formed in appreciable quantities when either phenylalanine or cinnamic acid was administered. Smaller amounts of *p*-coumaryl-quinic and chlorogenic acids were also synthesized. Although radioactive *p*-coumaric acid was identified in each experiment, phloretic acid was never labeled. The significance of these results in relation to the biogenesis of phloridzin in *Malus* is discussed.

Introduction

In a recent study in this laboratory (1) it was shown that the dihydrochalcone glucoside, phloridzin (Fig. 1), may be synthesized in *Malus* leaf disks from administered phenylalanine. Possible intermediates in this conversion of

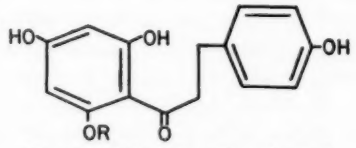

FIG. 1. Phloridzin. R = glucosyl.

phenylalanine to the $C_6(B)$–C_3 moiety of phloridzin are phenylpyruvic, phenyllactic, cinnamic, *p*-hydroxycinnamic, and phloretic (*p*-hydroxydihydrocinnamic) acids. The conversion of phenylalanine to cinnamyl-quinic and to *p*-coumaryl-quinic acids has been demonstrated recently by Levy and Zucker (2) and cinnamic acid-C^{14}, in turn, has been shown to be converted to hydroxylated phenylpropanoid compounds (3, 4, 5), quercetin (6), and lignins (7). The accumulating evidence indicates clearly that cinnamic acid is an intermediate in the biogenesis of plant polyphenols. It was of interest, therefore, to establish that cinnamic acid could be converted to phloridzin and to determine whether the fate of cinnamic acid paralleled that of phenylalanine. This report is concerned not only with the successful demonstration of the incorporation of cinnamic acid into the $C_6(B)$–C_3 moiety of phloridzin but also with the partial identification of some compounds common to both phenylalanine and cinnamic acid metabolism in *Malus*.

[1]Manuscript received November 25, 1960.
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Materials and Methods

Plant Material

Shoots or leaves of *Malus robusta*, *Malus baccata*, and *Malus mandshurica* were obtained from plants growing either in the greenhouse or on the grounds of the University. Most of the experiments were carried out with leaf disks, 1.2 cm in diameter, obtained with a Ganong leaf punch.

Radioactive Compounds

Cinnamic acid-2-C^{14}, L-phenylalanine-U-C^{14}, aspartic acid-U-C^{14}, and glucose-U-C^{14} were purchased from Merck and Co., Ltd., Montreal.

Administration of Radioactive Compounds and C^{14} Techniques

The methods of feeding radioactive compounds and the subsequent extraction and chromatography of extracts have been described (1, 8). Briefly, they were as follows: Leaf disks, after vacuum infiltration with water, were maintained in radioactive solutions in small petri dishes. Shoots were fed through their cut ends. Ethanolic extracts were chromatographed in phenol–water (4:1) followed by butanol – acetic acid – water (4:1:2.2). Phenolic acids were extracted successively with ether, 1% Na_2CO_3, and ether. The final ether extracts were chromatographed two-directionally in benzene – acetic acid – water (6:7:3) followed by sodium formate – formic acid – water (10:1:200). Phenolic acids were identified by their fluorescence characteristics and color reactions (9). Ultraviolet absorption spectra of cinnamic, *p*-coumaric, and phloretic acids were determined with a Beckman model DU spectrophotometer. The quinic esters of *p*-coumaric and caffeic acids, *p*-coumarylquinic and chlorogenic acids, respectively, were identified on chromatograms of ethanol extracts by their characteristic fluorescence behavior. Phloridzin was identified on the same chromatograms by means of its red fluorescence in ultraviolet light after exposure to ammonia fumes or with Ehrlich reagent with which it gives a red color on heating the chromatogram (10).

Keto acids were analyzed by chromatography of their 2,4-dinitrophenylhydrazones and by chromatography of the amino acids obtained on hydrogenolysis of these derivatives (11). Conversion of keto acid hydrazones to amino acids was found to be essential since phenolic acids are 'contaminants' of purified hydrazone fractions obtained from plant materials.

Radioactive spots were counted directly on paper chromatograms after location of the spots by radioautography using Kodak 'No-Screen' X-ray Film. The instrument used was a Nucleonics type IWAAA thin end-window (1.25 mg/cm^2) Geiger–Mueller tube attached to a Berkeley decimal scaler. Accurate radioactivity determinations of insoluble residues and total ethanol extracts were carried out by oxidation of aliquots to $^{14}CO_2$ with van Slyke reagent and measurement of the radioactivity with a Nuclear Chicago Model 600 dynamic condenser electrometer (Dynacon) incorporating an ion chamber. Samples were combusted in duplicate. This method was also used for phloridzin, in one series of experiments in which radioactive spots were cut out, eluted for 5 hours

with ethanol in micro-Soxhlets, and the eluates combusted after removal of the solvent.

Experimental and Results

Administration of Cinnamic Acid-2-C^{14}

A large number of experiments was carried out in which labeled sodium cinnamate was administered to leaf disks or to cut shoots before it was discovered that radioactive phloridzin could be obtained only when young leaf material (less than 6 weeks old) was used. Table I shows that with young leaf

TABLE I

Formation of radioactive phenolic compounds in *Malus* tissues from administered sodium cinnamate-2-C^{14}*

Expt. No.	Plant material	Time (hours)	Light or dark	% distribution of radioactivity in 80% ethanol-soluble fraction				
				Phloridzin	No. 1	No. 2	PQ†	Chlorogenic
1	*M. baccata* disks (M)	6	Dark	0	16	0	0	0
2	*M. baccata* disks (M)	27	Light	0	62	25	11	2
3	*M. baccata* shoots (Y)	24	Light	6	49	26	0	2
4	*M. robusta* disks (Y)	24	Light	20	49	14	4	1
5	*M. baccata* disks (Y)	4	Dark	16	34	7	0	0
	M. baccata disks (Y)	24	Dark	12	20	14	2	0
6	*M. mandshurica* disks (Y)	24	Light‡	2	37	15	4	3
	M. mandshurica disks (Y)	24	Light	2	29	20	5	2

NOTE: (M) = Disks obtained from mature leaves over 6 weeks old.
(Y) = Disks obtained from young leaves.
*Specific activity of sodium cinnamate, 6.5 μc/mg; 0.4 to 0.6 mg was administered to 10 disks in each treatment.
†*p*-Coumaryl-quinic acid.
‡0.1% sucrose administered together with cinnamic acid-C^{14}.

disks phloridzin-C^{14} was formed in a relatively short period, i.e., even in 4 hours. Table II shows the distribution of radioactivity between the ethanol-soluble and ethanol-insoluble fractions of leaf disks administered cinnamic acid-C^{14} and the percentage of total activity incorporated into phloridzin in other experiments.

Phloridzin-C^{14} was eluted from two-directional chromatograms with cold 95% ethanol and hydrolyzed on the steam bath with $Ba(OH)_2$. The $C_6(B)$–C_3 moiety, which is obtained as phloretic acid by this procedure, was identified by chromatography as being the only radioactive product. The specific radioactivity of phloridzin was obtained in one experiment by determining the concentration in an eluate (λ_{max} at 283 mμ in 95% ethanol) and combustion of the sample. It was found that the specific activity of phloridzin (92 μc/mmole) was approximately one-tenth that of the administered cinnamic acid (962 μc/mmole).

TABLE II

Percentage of conversion of cinnamic acid-2-C^{14} to phloridzin in leaf disks of *Malus baccata* in light

Material	Period of administration (hours)	Radioactivity (μc)*				% of total activity in phloridzin
		Administered	Recovered			
		Sodium cinnamate	Phloridzin	Ethanol solubles†	Insoluble residue	
10 disks of 4-day-old leaves	5	2.03	0.05	0.34	0.04	13
10 disks of 4-day-old leaves	17½	2.03	0.09	0.49	0.18	13
10 disks of 4-day-old leaves	24	2.03	0.07	0.52	0.26	9
21 disks of 10-day-old leaves	18	3.04	0.05	1.56	0.51	2.5
15 disks of 31-day-old leaves	18	3.60	0.17	2.90	0.59	2.0

*Determined after combustion, with Dynacon.
†Including phloridzin.

In every experiment an aliquot of the ethanol-soluble fraction was analyzed for free phenolic acids and for phenolic acids obtained after acid hydrolysis. Two-directional chromatograms showed that cinnamic, *p*-coumaric, and sometimes ferulic acid were the only radioactive acids present. Phloretic acid, a conspicuous phenolic acid in *Malus* leaves, was never radioactive.

Chromatograms of ethanol extracts showed that the quercetin and kaempferol glycosides (12) were never radioactive in older leaf tissues and only very slightly so (<1%) when 1-week-old leaves were used. *p*-Coumaryl-quinic acid (18, 19), identified by comparison with an authentic sample obtained through the courtesy of Dr. A. C. Hulme, Ditton Laboratories, U.K., was usually radioactive (Table I).

The most radioactive compound on chromatograms of ethanol extracts of leaf disks administered cinnamic acid-C^{14} was designated compound 1 (see Table I and Fig. 2). Repeated chromatography of this substance in butanol–acetic and the formate solvent followed by elution with cold 80% ethanol gave a purified extract which showed λ_{max} at 275 mμ in 80% ethanol, shifting to 279 mμ after the addition of 2 drops of 2 *N* NaOH. This compound did not give color reactions with either diazotized *p*-nitroaniline or diazotized sulphanilic acid and was not fluorescent in U.V. light, even after exposure to NH_3 fumes. Radioactive compound 1 was hydrolyzed with 1.5 *N* HCl for 3 hours on the steam bath and the hydrolyzate extracted continuously with ether. Chromatography of the ether extract in either the benzene–acetic solvent or *t*-amyl alcohol – ethanol – water (9:1:4) gave one radioactive compound identical in chromatographic behavior with cinnamic acid and readily distinguished from *p*-coumaric, phloretic, or ferulic acids. The U.V. absorption spectrum was identical with that of authentic cinnamic acid (λ_{max} at 273 mμ in 80% ethanol) eluted from the same chromatograms. The formation, in certain plant tissues,

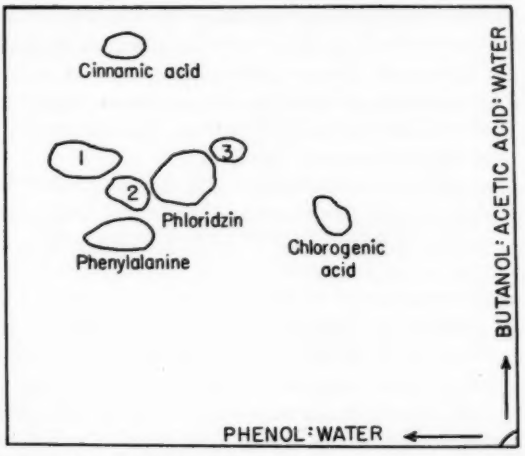

FIG. 2. Diagram of a two-directional chromatogram showing relative portions of some compounds synthesized from phenylalanine and cinnamic acid in *Malus*. (1) Cinnamyl glucose, (2) *p*-coumaryl glucose, (3) *p*-coumaryl-quinic acid.

of indoleacetyl aspartic acid from administered indoleacetic acid (13) led us to believe that compound 1 was the aspartyl derivative of cinnamic acid. Table V shows that when cinnamic acid together with aspartic acid-U-C^{14} was fed to disks, radioactive compound 1 was synthesized. However, acid hydrolysis of compound 1 obtained in this experiment produced a radioactive compound which was resolved from aspartic acid on two-directional chromatography. From its chromatographic behavior the compound resembled glucose. In this experiment considerable activity was detected in the sugars of the ethanol extract. The possibility that the other moiety of compound 1 was glucose and not aspartic acid was examined by feeding a young shoot of *Malus baccata* through the cut end with 400 μg of glucose-U-C^{14} (15 μc) together with 12 mg of non-radioactive sodium cinnamate. The 80% ethanol extract of leaves and stem was chromatographed repeatedly in butanol–acetic and phenol solvents to give a purified band of compound 1. Aliquots of the final eluate were hydrolyzed with (*a*) 1.5 *N* HCl on the steam bath, (*b*) 2 *N* NaOH in the cold, and (*c*) emulsin, and the hydrolysis products co-chromatographed with authentic glucose-C^{14}, aspartic and quinic acids. The major radioactive compound in the hydrolyzates was chromatographically identical with glucose. There were two minor radioactive contaminants which accounted for about 5% of the total activity.

In view of the very small amounts of compound 1 obtained by these methods it was not possible to crystallize it. The preliminary evidence accumulated here indicates that it is an ester of glucose and cinnamic acid. Because of the small amounts obtained it was not possible to determine the ratio of cinnamic acid to glucose in this compound.

A second prominent radioactive compound in ethanol extracts of leaf disks which had been administered cinnamic acid-C^{14} has been designated compound 2. Although this compound did not give color reactions of phenols, possibly due to the low amounts on chromatograms, it gave a blue fluorescence after exposure of the chromatograms to NH_3 fumes. This reaction is characteristic of *p*-coumaric acid and *p*-coumaryl-quinic acid.

Radioactive compound 2, obtained from cinnamic acid-2-C^{14} administration to leaf disks, was purified by chromatographing twice in phenol–water and in butanol – acetic – water followed finally by chromatography in 2% formic acid. In the last solvent the compound was resolved into two spots, a characteristic of *p*-coumaric acid and some of its derivatives and due to cis–trans isomerization (14). Elution of radioactive compound 2 with cold ethanol was followed by hydrolysis with either HCl, alkali, or emulsin. Hydrolyzates were extracted with ether and the ethereal extracts chromatographed in the benzene–acetic solvent together with standard phenolic acids. *p*-Coumaric acid-C^{14}, the only radioactive compound detected, was identified by (*a*) its blue fluorescence in the presence of NH_3 fumes, (*b*) its absorption spectrum (λ_{max} at 311 mμ), and (*c*) its identical chromatographic behavior with that of *p*-coumaric acid, e.g., formation of two spots (cis–trans isomers) in the formate solvent. Compound 2 was easily distinguished from either *p*-coumaric acid or *p*-coumaryl-quinic acid in all the solvents used and was synthesized by leaf tissue when *p*-coumaric acid was administered together with glucose-U-C^{14}. Elution of the compound followed by hydrolysis and chromatography yielded *p*-coumaric acid and glucose-C^{14} which were both identified by chromatography. Quinic and aspartic acids were run as standards and were shown to be absent from the products of hydrolysis. Compound 2 is therefore a conjugate of *p*-coumaric acid and glucose and could be either a glucoside or an ester. *p*-Coumaryl-quinic acid, an ester in which the phenolic hydroxyl of *p*-coumaric acid is free, fluoresces with NH_3 whereas *p*-coumaryl benzoate, in which the phenolic hydroxyl participates in the ester linkage, does not. Since compound 2 fluoresces with NH_3 in the presence of U.V. light this would suggest that it has a free phenolic hydroxyl group thus making it an ester. Corner and Harborne (15) have identified a *p*-coumaryl ester of glucose as a natural constituent of potato berries and have shown that the same compound is formed in tissues of higher plants fed with *p*-coumaric acid (16). The *p*-coumaryl glucose ester of Corner and Harborne showed a λ_{max} at 312 mμ shifting to 365 mμ on the addition of NaOH. Compound 2 showed a λ_{max} at 314 mμ shifting to 368 mμ with alkali. Both isomers of compound 2 which were obtained with the formate solvent had identical absorption spectra. Jurd (17) has shown that methyl *p*-coumarate shows a λ_{max} at 313 mμ in ethanol shifting to 368 mμ in ethanol/sodium ethoxide so that the absorption spectra of compound 2 are consistent with those of a *p*-coumaryl ester. Both Jurd and Corner and Harborne have identified peaks at shorter wavelengths for these *p*-coumaryl compounds but it was not possible to determine whether these were characteristic of compound 2 due to the high absorp-

tion of eluates from paper chromatograms in this region of the spectrum.

Administration of Phenylalanine-C^{14}

Disks of *M. baccata* or *M. mandshurica* were administered uniformly labeled L-phenylalanine in three different experiments, the details and results of which are recorded in Table III. As with cinnamic acid-C^{14}, only young leaf material

TABLE III

Formation of radioactive phenolic compounds in *Malus* leaf disks* from administered phenylalanine-U-C^{14}

Plant material	Time (hours)	Light or dark	% distribution of radioactivity† in 80% ethanol fraction				
			Phloridzin	No. 1	No. 2	PQ‡	Chlorogenic
M. baccata (mature)	24	Light	0	5	5	0	0
M. baccata (mature)	6	Dark	0	9	2	Trace	Trace
M. baccata (mature)	9	Dark	0	17	1	Trace	Trace
M. baccata (mature)	12	Dark	0	26	0	Trace	Trace
M. baccata (mature)	22	Dark	0	27	0	Trace	Trace
M. baccata (mature)	30	Dark	0	32	0	Trace	Trace
M. mandshurica (young)	24	Light	5	8	7	19	11
M. mandshurica (young)	24	Light	5	13	8	8	9

*Ten disks per treatment. Specific activity of phenylalanine-C^{14}, 3 μc/mg.
†Radioactivity determinations made by direct counting of spots on paper.
‡*p*-Coumaryl-quinic acid.

was found to be capable of effecting a synthesis of phloridzin from phenylalanine. Compounds 1 and 2, *p*-coumaryl-quinic and chlorogenic acids, which were prominent in the cinnamic acid experiments, were major radioactive compounds in these experiments. Hydrolysis studies and co-chromatography showed that they were identical with the compounds formed from cinnamic acid. Aliquots of ethanol extracts were hydrolyzed with acid and the phenolic acid fraction, i.e., ether- and bicarbonate-soluble fractions, analyzed by two-directional chromatography as before. Radioactive cinnamic, *p*-coumaric, and ferulic acids were the only radioactive compounds detected. Phloretic acid was never radioactive.

In a fourth experiment, a solution of phenylalanine-C^{14} was administered to 45 leaf disks for 16½ hours at the end of which time nine disks were killed and analyzed and the remainder transferred, after being washed, to tap water in the dark. Samples of nine disks were removed and killed after intervals of 3, 6, 11¼, and 25 hours. Aliquots of the 80% ethanol-soluble fractions were combusted in duplicate and radioactivity determinations made using the Dynacon. The insoluble residues remaining after extraction of the disks with ethanol were also combusted and assayed for radioactivity. Chromatograms and radioautographs of ethanol extracts were prepared and radioactive spots were counted directly on the paper. The results of this experiment are presented in Table IV. They show that phloridzin-C^{14} and, to a much lesser extent, chlorogenic acid, accumulated with time and that the level of phenylalanine-C^{14}

TABLE IV

Changes with time in the distribution of activity in *Malus baccata* leaf disks administered phenylalanine-U-C^{14}* in the dark

Treatment	Radioactivity in:†			Distribution of radioactivity‡ in 80% ethanol-soluble fraction (mμc)					
	Ethanol-soluble fraction (μc)	Insoluble residue (μc)	Total (μc)	Phenylalanine	Phloridzin	No. 1	No. 2	PQ§	Chlorogenic acid
16½ hours in phenylalanine-C^{14}	0.31	0.27	0.58	73	18	36	25	14	12
16½ hours in phenylalanine-C^{14} +3 hours in water	0.24	0.25	0.49	29	26	22	24	7	12
16½ hours in phenylalanine-C^{14} +6 hours in water	0.21	0.24	0.45	15	34	32	8	3	10
16½ hours in phenylalanine-C^{14} +11¼ hours in water	0.24	0.23	0.47	18	50	24	14	0	19
16½ hours in phenylalanine-C^{14} +25 hours in water	0.21	0.26	0.47	11	41	25	0	0	17

*3 μc of phenylalanine-C^{14} (specific activity, 10 mc/mmole) administered to 45 disks; 9 disks per treatment.
†Radioactivity determined by combustion.
‡Radioactivity determined by direct counting of spots on paper.
§*p*-Coumaryl-quinic acid.

dropped. In this experiment a number of other radioactive compounds was detected on chromatograms but these were not studied because of their low activities.

TABLE V

Formation of radioactive phenolic compounds from administered glucose-C^{14} and aspartic-C^{14} in *Malus* leaf disks*

Compounds administered	Material	Treatment	% distribution of radioactivity† in 80% ethanol-soluble fraction				
			Phloridzin	No. 1	No. 2	PQ‡	Chlorogenic
Glucose-U-C^{14} +*p*-coumaric	*M. baccata* (mature)	24 hours, light	5	0	16	0	0
Glucose-U-C^{14} +cinnamic	*M. baccata* (mature)	24 hours, light	3	20	3	0	0
Aspartic-U-C^{14} +cinnamic	*M. baccata* (young)	24 hours, light	3	18	3	0	0

*2 μc radioactive compound in 0.5 ml water and 0.5 ml of 10 mmoles cinnamic or *p*-coumaric acids fed to sets of 10 leaf disks.
†Radioactivity determinations made by direct counting of spots on paper.
‡*p*-Coumaryl-quinic acid.

The possibility that one of these compounds was phenylpyruvic acid-C^{14} was studied in the following way: 80 μg of phenylalanine-U-C^{14} with an activity of 2.5 μc was administered to a set of 10 disks for 24 hours in the light. A second set of disks was fed the same amount of radioactive compound together with 1.6 mg of phenylpyruvic acid. Chromatograms of 2,4-dinitrophenylhydrazones suggested the presence of phenylpyruvic dinitrophenylhydrazone-C^{14} but phenylalanine-C^{14} was not detected among the hydrogenolysis products. This experiment was repeated and it was shown once more that no phenylpyruvic-C^{14} had been formed from administered phenylalanine-C^{14}.

Discussion

Radioactive phenylalanine or cinnamic acid has been administered to various plant materials usually for the purposes of studying incorporation into specific compounds. As far as we are aware no attempts have been made so far to demonstrate, by chromatography, the patterns of ethanol-soluble compounds synthesized in any one plant tissue from either of these substances. Although the method of estimating radioactivity in spots on chromatograms, as adopted in most of the experiments reported here, is relatively crude, it is of considerable value since it provides for a rapid analysis of the fate of a particular radioactive compound introduced into a plant tissue.

The fate of both cinnamic acid and phenylalanine in *Malus* leaf tissue is complex. The nature of the radioactive ethanol-insoluble material obtained with phenylalanine-C^{14} and accounting for 50% of the total activity taken up by disks (see Table IV) was not determined. The labeled insoluble material from the cinnamic-C^{14} experiments (see Table II) is presumed to be lignin. If this is the case these results indicate that lignification may proceed in

isolated leaf tissue. Alkaline nitrobenzene oxidations of lignin would not have been useful as the hydroxybenzaldehydes, which would have been produced in this reaction, would have been non-radioactive since cinnamic-2-C^{14} was used.

Only a small proportion of the total ethanol-soluble activity was located in phloridzin after the administration of either phenylalanine or cinnamic acid-C^{14}, and this could be demonstrated only with young leaves. Failure of older leaf material to utilize either phenylalanine or cinnamic acid in phloridzin synthesis cannot be explained at the present time. Metabolic changes in ageing leaves are well known and it would appear now that recognition of this factor is essential to the study of the biogenesis of certain phenolic compounds. This was shown to be the case in *Pyrus communis* (20) where it was found that although chlorogenic acid synthesis from phenylalanine continued in old leaves, arbutin, the glucoside of hydroquinone, was formed only in young leaves. Quantitative changes of the phenolic compounds in leaves during the course of a season's growth (21, 22) support the concept of a changing pattern of aromatic metabolism with maturation.

Among the major radioactive compounds synthesized in *Malus* from administered labeled phenylalanine and cinnamic acid were cinnamyl and *p*-coumaryl conjugates of glucose. Although these compounds await isolation and complete chemical identification we consider it of importance to indicate their nature at this stage of our investigations. Levy and Zucker (2) have demonstrated recently that compounds with the characteristics of cinnamyl and *p*-coumaryl esters of quinic acid are formed from administered phenylalanine-C^{14} in potato tissues. Harborne and Corner, on the other hand, have indicated that a *p*-coumaryl ester of glucose is formed in tissues of higher plants fed with *p*-coumaric acid (16). Andreae and Good (13) have shown that another aromatic acid, indoleacetic acid, forms an aspartyl derivative when introduced into plant tissues. In *Malus* the glucose derivatives of cinnamic and *p*-coumaric acids are the most radioactive ethanol-soluble compounds synthesized from either phenylalanine or cinnamic acid. It should be emphasized here that chromatograms of *Malus* extracts, from phenylalanine and cinnamic acid feedings, were essentially similar in that the same radioactive compounds were present.

Whether compounds 1 and 2, which have been tentatively identified as esters of glucose and cinnamic or *p*-coumaric acids, are intermediates or whether they are stable end products of metabolism must await further studies. They may be artifacts or 'detoxication' products which bear no relation to the process of phloridzin biogenesis in *Malus*. For example, salicin, the β-D-glucoside of salicyl alcohol, occurs naturally in a number of plant species but when salicyl alcohol is administered to plants (*o*-hydroxybenzyl)-β-glucoside, which has not been found to occur naturally, is synthesized (23). Similarly, *Malus* tissues can be induced to form novel glycosides from administered phenols (24). It has not been possible as yet to determine whether compounds 1 and 2 are normal constituents of *Malus* leaf tissue.

The identification of phloretic acid among the phenolic acids of *Malus* tissues is of particular interest in view of its structural relationship to the $C_6(B)$–C_3 moiety of phloridzin. Phloretic acid was never labeled in any of the experiments reported here even in those cases where phloridzin-C^{14} was synthesized. In contrast, free and bound *p*-coumaric acid was radioactive in all experiments, even when phloridzin-C^{14} was not formed. Radioautographs and chromatograms showed that the phenolic acid fraction obtained after acid hydrolysis of ethanolic extracts always contained radioactive *p*-coumaric, but never phloretic, acid. This would suggest that phloretic acid or derivatives of it are not in the pathway of phloridzin synthesis. Phloretic acid in *Malus* may be formed as a result of phloridzin hydrolysis in the same way as it may be produced when certain fungi are grown in the presence of phloridzin (25, 26) although it then becomes difficult to explain the presence of this acid in *P. communis* (9) which does not contain phloridzin. This question could be resolved with the administration of phloretic acid-C^{14}.

Phenylpyruvic acid has not been detected in plant tissues (27) and in view of the failure to detect it in *Malus* leaf tissues it seems unlikely that phenylalanine is converted to cinnamic acid or its derivatives through reactions such as a direct oxidation or by transamination to phenylpyruvic acid. Kuokol and Conn (28) have described an enzyme from plants, phenylalanase, which catalyzes the formation of cinnamic acid from phenylalanine. This enzyme may be operative in *Malus* tissues.

The formation of cinnamyl and hydroxycinnamyl compounds from phenylalanine does not appear to be reversible. This suggests that the levels of phenylalanine in leaf tissues determine the levels of those phenolics with a phenylpropanoid component in the carbon skeleton. Conditions favorable for the hydrolysis of protein in leaves, such as excision or shortage of water, would be expected to result in increased synthesis of phenolics. This possibility should be examined.

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THE PLASMA 17-HYDROXYCORTICOSTEROID LEVELS IN ACUTE AND CHRONIC RENAL FAILURE[1]

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Abstract

The free and glucuronide fractions of the plasma 17-hydroxycorticosteroids have been determined on specimens from 14 adult controls, 7 patients with chronic renal disease, and 11 patients with acute renal failure. The results have been statistically analyzed. Both free and glucuronide fractions were shown to be elevated in cases of acute renal failure while neither was significantly altered in the cases of chronic renal disease. The changes in these fractions during diuresis and hemodialysis in a few cases of acute renal failure are also reported. The possible mechanisms for these findings are briefly discussed.

Introduction

Patients with chronic renal disease have been shown by Englert *et al.* to have normal plasma levels of free 17-hydroxycorticosteroids but an elevation of the conjugated levels correlating inversely with the endogenous creatinine clearance (1). These workers also were able to demonstrate that the disappearance rate of the free 17-hydroxycorticosteroids from the plasma, following the intravenous administration of cortisol, was prolonged over that of normal controls and they concluded that the rate of corticosteroid reduction and conjugation was impaired in uremia.

This report consists of a study of the plasma concentrations of both the free and the conjugated 17-hydroxycorticosteroids (17-OHCS) in patients with acute or chronic renal failure. Observations were also made during the diuretic phase of recovery from acute renal failure as well as during and after extracorporeal hemodialysis.

Methods

Heparinized venous blood was drawn from 14 adult controls and 7 patients with chronic renal failure at approximately 8.00 a.m. after an overnight fast. Similar specimens from 11 patients with acute renal failure were usually drawn in the morning; however, some were drawn in the afternoon or early evening. The blood was rapidly separated by centrifugation and the plasma was stored in the frozen state.

Normal controls consisted of medical students, graduate nurses, house officers, and convalescing patients with no evidence of endocrine, hepatic, or renal disease. The patients with chronic renal failure had various diseases as

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[2]A portion of this work was carried out during the tenure of a Ciba Medical Research Fellowship.

[3]Markle Scholar in the Medical Sciences.

TABLE I

Plasma 17-hydroxycorticosteroid and 17-hydroxycorticosteroid glucuronide content in subjects with normal renal function

Subject No.	Sex	Age	Free 17-OHCS (μg/100 ml)	17-OHCS glucuronide (μg/100 ml)
1	M	72	8.0	46.5
2	M	74	10.0	15.0
3	M	27	19.5	9.0
4	M	24	7.5	25.0
5	M	55	20.0	*
6	M	65	7.0	*
7	M	24	30.5	9.0
7	M	24	17.5	3.0
7	M	24	21.5	10.0
7	M	24	21.0	*
7	M	24	19.0	9.0
8	M	55	16.6	24.1
9	M	35	10.5	4.7
10	M	51	21.7	3.7
11	M	34	13.0	10.0
11	M	34	12.5	6.0
12	F	30	19.5	104.0
13	F	25	20.9	*
14	F	28	10.4	*
Mean			16.1	19.9
±S.D.			7.8	53.8

*High background, values probably less than 10 μg/100 ml.

listed in Table II. The various causes of acute renal failure are listed in Table III; all of these patients were anuric or severely oliguric for variable periods of time. Repeat determinations were made in three patients during the diuretic phase and in three patients during and after extracorporeal hemodialysis with the Kolff, twin-coil, artificial kidney.

The free 17-hydroxycorticosteroids were determined according to the method of Silber and Busch (2). The glucuronide fraction was determined as follows: Subsequent to the extraction of the free fraction, the aqueous phase was adjusted to a pH of 4.5 with 5% sulphuric acid; acetate buffer, pH 5 in a volume equal to that of the aqueous phase, was added. Beef liver beta-glucuronidase was added, 100 units every 2 hours for 3 times, and the aqueous phase was incubated at 37° C for 24 hours. Following this, the aqueous phase was extracted with methylene dichloride and the entire process of incubation and extraction was repeated. The extracts were combined and the 17-hydroxy-corticosteroid glucuronide content determined according to the Silber–Busch method (2).

Results

The values for the plasma concentration of the free and conjugated 17-OHCS in the normal controls are listed in Table I. The free 17-OHCS had a mean of 16.1 (S.D. ± 7.8) μg/100 ml with a range of 7.0 to 30.5 μg/100 ml; the conjugated fraction had a mean of 19.9 (S.D. ± 53.8) μg/100 ml with a range of 3.0 to 104.0 μg/100 ml. The values for these fractions in the seven patients with

TABLE II

Plasma 17-hydroxycorticosteroid and 17-hydroxycorticosteroid glucuronide content in cases with chronic renal disease

Patient No.	Diagnosis	Sex	Age	NPN (mg/100 ml)	Free 17-OHCS (mg/100 ml)	17-OHCS glucuronide (mg/100 ml)
1	Chronic glomerulonephritis	M	42	1	11.6	31.8
2	Arteriolar nephroscelerosis	M	70	100	18.1	109.8
2	Arteriolar nephroscelerosis	M	70	100	16.2	174.0
3	Chronic glomerulonephritis	M	25	100	3.6	29.8
3	Chronic glomerulonephritis	M	25	144	8.4	22.9
4	Diabetic glomerulosclerosis	F	55	133	14.6	69.7
5	Gout with nephropathy	M	60	200	18.4	7.5
6	Probable pyelonephritis	F	37	50	15.4	21.6
7	Probable pyelonephritis	F	71	*	27.0	33.7
Mean					14.8	55.6
±1 S.D.					6.6	54.0
P value					$0.70 > P > 0.60$	$0.20 > P > 0.10$

*Note determined at this time; previously the NPN had been repeatedly elevated.

TABLE III

Plasma 17-hydroxycorticosteroid and 17-hydroxycorticosteroid glucuronide content in cases with acute renal failure

Patient No.	Diagnosis	Sex	Age	NPN* (mg/100 ml)	Free 17-OHCS (μg/100 ml)	17-OHCS glucuronide (μg/100 ml)
1	Acute renal failure, undetermined etiology	F	28	192	25.0	321.0
2	Acute tubular necrosis, circulatory collapse	M	61	180	33.0	208.0
3	Acute tubular necrosis, circulatory collapse	M	59	197	22.7	154.0
4	Acute tubular necrosis, circulatory collapse	M	50	102	77.5	111.5
5	Acute tubular necrosis, circulatory collapse	M	46	293	59.0	604.0
6	Acute renal failure, undetermined etiology	M	42	†	93.7	766.0
7	Acute exaccerbation of glomerulonephritis	F	42	240	16.6	107.1
8	Acute exaccerbation of chronic pyelonephritis	F	26	240	76.5	441.0
9	Renal cortical necrosis	M	46	265	24.4	209.0
10	Acute tubular necrosis, circulatory collapse	M	30	115	33.0	329.0
11	Acute tubular necrosis, transfusion reaction	F	49	195	64.5	930.0
Mean					47.8	380.1
±1 S.D.					27.0	277.5
P value					$0.01 > P > 0.001$	$0.01 > P > 0.001$

*NPN as determined at the time of sampling for plasma steroids.
†Not determined at the time of sampling.

chronic renal failure (Table II) were not significantly different from those of the normal controls; the mean for the free fraction was 14.8 (S.D. $\pm$6.6) μg/100 ml ($0.70 > P > 0.60$); the mean for the conjugated fraction was 55.6 (S.D. $\pm$54.0) μg/100 ml ($0.20 > P > 0.10$).

The free 17-OHCS concentration was elevated in 5 of the 11 patients of acute oliguric renal failure; the mean for the group was 47.8 (S.D. $\pm$27.0) μg/100 ml which is significantly greater than that of the normal controls ($0.01 > P > 0.001$). Likewise, the conjugated 17-OHCS concentration was grossly elevated in at least 9 of the 11 patients; the mean value was 380.1 (S.D. $\pm$277.5) μg/100 ml which is also significantly greater than that of the normal controls ($0.01 < P < 0.001$).

In the three patients who were studied through the diuretic phase the levels of both the free and conjugated 17-OHCS decreased during the diuresis (Table IV).

TABLE IV

Follow-up studies on three cases of acute renal failure during diuresis

Patient No.	Date	NPN (mg/100 ml)	Free 17-OHCS (μg/100 ml plasma)	17-OHCS glucuronide (μg/100 ml plasma)	Comment
1	3/24/58	192			
	3/28/58		25.0	321.0	
	4/ 1/58	274			
	4/ 2/58		17.0	289.0	
	4/ 5/58		32.5	280.0	
	4/ 6/58	228			Start of diuresis
	4/11/58	144			
	4/12/58		14.0	264.0	
	4/17/58	75			
	4/18/58		4.0	104.0	
	4/28/58	31			
	5/13/58		4.5	20.0	
7	12/19/58	240			
	12/28/58	279			
	12/31/58	268	16.6	107.1	
	1/ 3/59	225	9.4	54.7	
	1/ 8/59	147			Start of diuresis
	1/ 9/59	139	52.2	350.0	Cortisone, 250 mg
	1/13/59		40.8	335.0	Cortisone, 250 mg
	1/14/59	77			Cortisone, 250 mg
	1/16/59				Discontinued cortisone
	1/17/59	67			
	1/20/59	50	6.4	10.3	
	2/ 2/59		6.4	*	
	2/ 9/59	102	7.5	*	
	2/12/59	67	8.5	*	
10	2/ 6/59	363			
	2/ 7/59	325	33.0	329.0	
	2/10/59	328	11.9	327.0	Start of diuresis
	2/12/59		24.8	126.0	
	3/14/59	115			
	2/15/59	75			
	2/16/59		17.3	14.3	

*High background, values probably less than 10 μg/100 ml.

TABLE V

Follow-up studies on three cases of acute renal failure during hemodialysis

Patient No.	Date	NPN (mg/100 ml)	Free 17-OHCS (μg/100 ml plasma)	17-OHCS glucuronide (μg/100 ml plasma)	Comment
9	1/ 6/59	225			
	1/ 9/59	265	24.4	209.0	Prior to hemodialysis
	1/ 9/59	193	37.1	168.0	During hemodialysis
	1/10/59	151.5	35.7	165.0	Following hemodialysis
	1/10/59	121.5			Prior to hemodialysis
	1/10/59	106	29.1	139.0	Following hemodialysis
	1/11/59	206.5	19.5	321.0	
	1/16/59	215	14.2	225.0	Prior to hemodialysis
	1/17/59	171	18.8	194.0	Following hemodialysis
	1/19/59	171			
	1/22/59		13.0	136.0	Terminal
11	2/26/59	195	64.5	930.0	
	3/ 1/59	195	18.4	875.0	Prior to hemodialysis
	3/ 1/59	171	20.7	799.0	Following hemodialysis
	3/ 5/59		20.4	463.0	Diuresis
	3/ 7/59	307	9.7	360.0	
	3/ 9/59	348			Expired
12*	12/29/58	187			Hydrocortisone therapy daily
	1/ 2/59	279			
	1/ 3/59	276	78.4	774.0	
	1/ 6/59	328			
	1/ 8/59	368	50.3	694.0	Prior to hemodialysis
	1/ 9/59	106	57.5	614.0	Following hemodialysis

*Case with acute tubular necrosis secondary to a transfusion reaction.

In the three patients studied during and after extracorporeal hemodialysis the free fraction did not change significantly whereas the glucuronides showed a small to moderate decrease in concentration.

Discussion

The observation of markedly increased plasma levels of 17-OHCS in patients with acute, oliguric renal failure is certainly consistent with reasonable predictions. Since renal excretion is an important and well-established aspect of corticosteroid metabolism, the virtual cessation of renal function would certainly be expected to lead to accumulation of 17-OHCS in the extracellular fluid. Likewise, current concepts of the hepatic phase of corticosteroid metabolism would lead to the prediction that the glucuronide fraction would show the greatest increment of change; this prediction is supported by these observations. The increased levels of the free fraction may result from an increased hormone secretion rate, a decreased volume of distribution, a decreased or limited rate of hepatic reduction and conjugation, or any combination of these three factors. Our data do not shed any light on this problem, and little is known of corticosteroid metabolism in uremia. Whatever the causes for these elevated plasma levels are, it is evident that they are reversible as the renal failure improves, as is shown in the follow-up studies on three patients with

acute renal failure during diuresis. It seems reasonable to speculate that the apparent "catabolic response" of patients with acute renal failure may well be due, at least in part, to the increased levels of free 17-OHCS in the extracellular fluid.

We cannot explain the exact mechanism whereby the levels of free and conjugated plasma 17-OHCS are maintained within the normal range in patients with chronic, azotemic, renal failure. The reasonable possibilities include a renal mechanism and an alteration in the extrarenal metabolism of the corticosteroids.

With regard to the former, available evidence suggests that the renal handling of the free 17-OHCS consists of glomerular filtration of the non-protein-bound portion followed by variable tubular reabsorption (3–8). There is some evidence that, under some conditions, the free fraction may be secreted by the renal tubules, but this is far from established (8). The non-protein-bound portion of the conjugated fraction is also filtered; subsequently, there is net tubular reabsorption or secretion (3–6, 8) but the factors determining net tubular transport have not been elucidated.

There can be no doubt that the glomerular filtration rate and renal plasma flow were markedly diminished in all of the patients studied. There are three possible ways in which excretion of the 17-OHCS might be maintained at, or near, normal levels despite a marked reduction of the GFR. First of all, the extent to which the various plasma fractions are bound to protein may be reduced so that the filterable fractions are increased and the filtered loads of 17-OHCS are kept within the normal range. Secondly, the extent to which the filtered 17-OHCS are reabsorbed by the tubules may be grossly reduced; with such an increase in the excretion fraction, excretion rates may be maintained. Thirdly, tubular secretion may occur and increase relatively to the decrease in filtered loads. The second possibility may occur as a result of "nephron osmotic diuresis".

On the other hand, it may be that the 17-OHCS secretion rate is reduced in chronic renal failure either as a direct result of the uremia, or as a result of diminished renal excretion leading to slight elevations of the free fraction with resultant "negative feed-back" suppression of ACTH secretion. A third possibility is the impairment of hepatic reduction and conjugation of the free fraction in uremia with resultant decrease in secretion rates; the observations of Englert *et al.* tend to support this view (1).

Clarification of the question will depend upon measurement of hormone turnover rates, studies of hepatic metabolism of 17-OHCS, and definitive studies of the renal handling of the 17-OHCS in uremic patients. Improved methods are certainly required if the latter factor is to be accurately assessed.

Sandberg and Slaunwhite (9) have shown that the glucuronides have less affinity for transcortin than do the free 17-OHCS. This could explain why the glucuronide fraction was demonstrably dialyzable during hemodialysis while the free fraction was not. Furthermore, hemodialysis per se may well have

resulted in an increased rate of adrenocortical hormone production; during the relatively short period of hemodialysis this would most likely be reflected in an increase of the level of the free fraction only. Should the blood volume have been contracted during hemodialysis, removal of plasma steroids may have been partially masked. Combinations of these factors, and possibly other unrecognized factors, probably account for these post-hemodialysis findings.

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NUCLEIC ACID SYNTHESIS AND TRANSFER IN NORMAL, SECOND GENERATION CHICK EMBRYO FIBROBLASTS[1]

R. Bather and Elizabeth Purdie-Pepper

Abstract

The stripping film technique of autoradiography has been used to study some aspects of RNA and DNA metabolism in chick embryo fibroblasts in second generation tissue culture.

Approximately one third of the cells incorporated thymidine-H^3 into DNA in a 20-minute uptake period. The duration of DNA synthesis, the generation time (time elapsing between two successive cell divisions), and the duration of mitosis have been calculated and are very similar to the values obtained for pure strains of hamster cells maintained in culture for several months by another author.

RNA synthesis, as measured by uridine-H^3 incorporation, occurred only in the nucleus and nucleolus. Both sites synthesized RNA simultaneously beginning at zero time. The ratio of the number of grains over the nucleolus to that over the whole nucleus remained constant throughout the uptake of uridine-H^3 and its transfer to the cytoplasm.

A small amount of labelled soluble RNA precursors remain in the nucleus after removal of uridine-H^3 from the medium. This results in a slight rise in radioactivity of the nucleus after uridine-H^3 removal. RNA then leaves the nucleus rapidly and appears in the cytoplasm. The half life of RNA in the nucleus is about 4 hours. Some turnover of cytoplasmic RNA seems to occur after 8 hours but quantitative estimates of its rate cannot be made due to changing geometry of the cells as the RNA migrates from the nuclear to the peripheral parts of the cell.

Finally, little or no RNA synthesis occurs for a period of about 30 minutes during contraction of the chromosomes in mitosis.

Introduction

Autoradiography using stripping film (1) and tritiated nucleic acid precursors has proved useful in following nucleic acid synthesis and transfer at the level of the individual cell. A number of reports have appeared in the recent literature describing such experiments on a variety of species of cell (2, 3, 4, 5, 6, 7).

We are interested in the possibility of using the autoradiographic technique for the in vitro study of virus synthesis in Rous sarcoma cells. It is now well established that normal chick embryo fibroblasts can be transformed into typical virus producing Rous sarcoma cells by infecting in vitro with Rous sarcoma virus (8, 9, 10, 11), and the malignant transformation which takes place has been observed many times in our own laboratory.

The present paper describes preliminary experiments on RNA and DNA synthesis in normal, second generation chick embryo fibroblasts of the type easily infected by Rous virus. Some of these results have been briefly reported elsewhere (12).

Materials and Methods

Nine-day-old chick embryos were trypsinized with 0.25% trypsin in balanced salt solution containing no divalent cations. Approximately 2×10^6 cells were

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Contribution from the Saskatchewan Research Unit of the National Cancer Institute of Canada, University of Saskatchewan, Saskatoon, Sask.

transferred to 2-oz bottles in a growth medium consisting of 90% synthetic medium 1066* and 10% calf serum. In 3–5 days, monolayers were formed which were mostly of fibroblast cells with scattered islands of epithelial cells. The monolayers were trypsinized (0.1% trypsin) and approximately 5×10^6 cells transferred to 100-mm Petri dishes containing several 11×22 mm cover slips. These cells were allowed to multiply for 24 hours in the growth medium in an atmosphere of air containing 5% CO_2. The epithelial cells failed to grow at the second transplanting and the Petri dishes contained only fibroblasts.

Tritium-labelled uridine (specific activity, 680 mc/mmoles) or tritium-labelled thymidine (specific activity, 1900 mc/mmoles), both at a level of 10 μc per ml, were fed to the cells in growth medium. After suitable periods of absorption, the radioactive medium was washed off and replaced with growth medium containing an excess of unlabelled nucleoside in order to dilute out the remaining radioactive precursors. Unlabelled uridine and cytidine were used to dilute labelled uridine, and unlabelled thymidine was used to dilute labelled thymidine, the former at 15–25 times and the latter at 50 times the concentration of the labelled nucleoside.

Cover slips were removed at required intervals, washed in three changes of Hanks' balanced salt solution, and fixed in methanol for 15 minutes. Nucleic acids were retained and soluble precursors washed from the cells by treatment with cold 5% trichloroacetic acid for 5 minutes and the cover slips were then washed 10 times in distilled water, dried, and cemented cell-side up on ordinary microscope slides with "Permount". The microscope slides had been previously dipped in a "subbing" solution consisting of 0.5% gelatin plus 0.05% chrome alum in distilled water and allowed to dry. This treatment helps to prevent the photographic film from leaving the glass during subsequent handling.

In some experiments ribonuclease treatment was employed at this stage. The enzyme was dissolved in saline (1 mg per ml) and the slides immersed in the solution for 2 hours at room temperature followed by several washings in saline.

Kodak AR 10 stripping film was applied to the slides and exposed for suitable periods of time (usually about 5 days). The slides were then developed in half-strength Kodak D-19 developer for 8 minutes followed by a quick rinse in water. Fixing time was 10 minutes in half-strength Kodak acid fixer followed by 5 minutes of rinsing in water. The film was cleared with half-strength Kodak hypo clearing agent for 2 minutes followed by rinsing in at least three changes of water over a 5-minute period. The cells were then stained through the film for 20 minutes with azure-B bromide, rinsed for a few seconds in distilled water, and allowed to dry in air at room temperature. It was found essential that all the photographic and staining solutions be made up fresh for each batch of slides.

Counts of grains in the emulsion overlying the nucleus, nucleolus, or cytoplasm in interphase cells or overlying the chromosomes and extrachromosomal

*Connaught Laboratories, Toronto, Ontario.

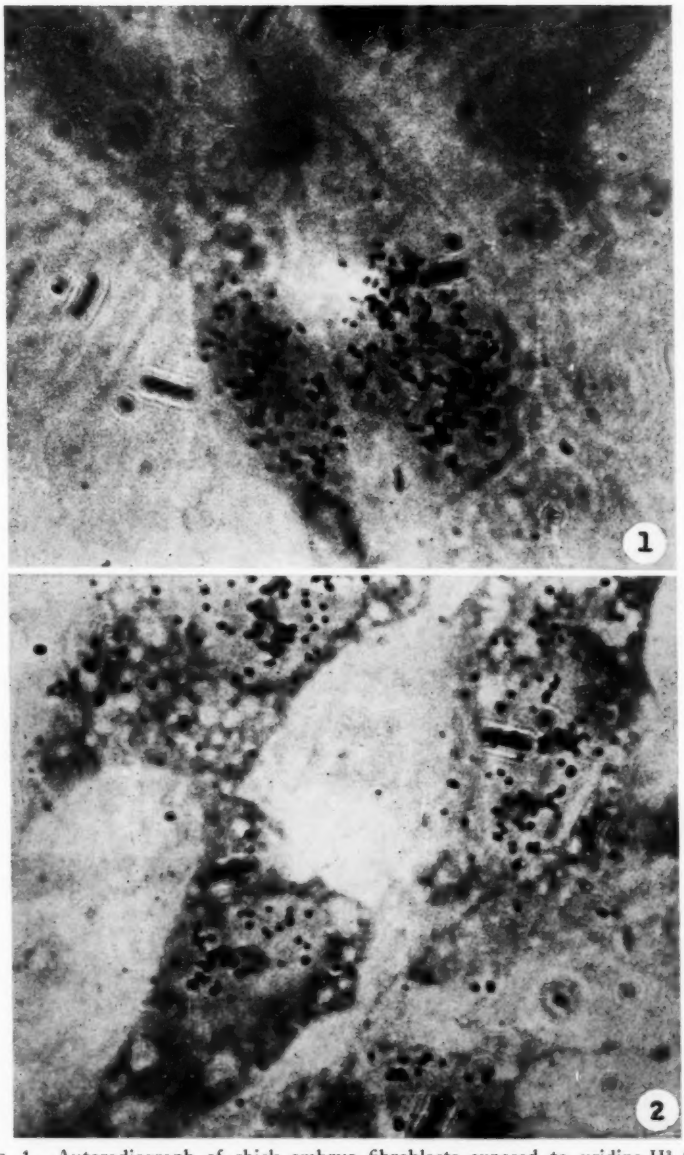

FIG. 1. Autoradiograph of chick embryo fibroblasts exposed to uridine-H^3 for 20 minutes before fixing. All emulsion grains are located over the nucleus. (×1500)

FIG. 2. Autoradiograph of chick embryo fibroblasts exposed in uridine-H^3 for 20 minutes then transferred to non-radioactive medium containing excess uridine and cytidine for 1 hour. Emulsion grains now appear in the cytoplasm. (×1500)

matrix in mitotic figures were made with the aid of an automatic tally when necessary. Corrections for background were made by counting emulsion grains in an eyepiece grid in cell-free areas adjacent to the counted cells. The area occupied by a cell or portion thereof was also estimated with the eyepiece grid and at least 10 times the area was counted for background. Further details of procedure are given in the results.

Results

Figure 1 shows chick embryo fibroblasts which have taken up tritium-labelled uridine for 20 minutes. All the emulsion grains in the film when corrected for background are located over the nucleus and nucleolus. Figure 2 shows fibroblasts 1 hour after removal of radioactive uridine. Grains now appear over both nucleus and cytoplasm. Background grain counts were always low, usually no more than one or two grains per nucleus. Counts of grains over 30 cells on each slide showed a standard error less than 10% of the mean.

Viability of Cells after Incorporation of Thymidine-H³

In view of reports of altered DNA metabolism and decreased cell viability following several hours of growth in the presence of tritium-labelled thymidine (13, 14), an experiment was done to test cell viability after short-term exposure to thymidine-H³. Monolayers of chick fibroblasts growing in Leighton tubes were exposed to thymidine-H³ for 20 minutes at levels of 0.1 μc/ml and 10 μc/ml. The radioactive medium was then replaced with growth medium containing excess thymidine. Duplicate Leighton tubes were sacrificed at zero time and 48 hours. The cells were trypsinized and resuspended in balanced salt solution containing erythrosin B for viability counts (15). The length of time between removal of growth medium and counting was standardized throughout.

Table I shows that the percentage of unstained cells (actively metabolizing cells) in the cultures which had taken up thymidine-H³ was not markedly different from that of the controls after 48 hours' growth, during which time

TABLE I

Percentage of erythrosin-unstained chick fibroblasts at intervals after 20 minutes' uptake of tritium-labelled thymidine

Time after removal of radioactive medium (hours)	Tritium content of radioactive medium (μc/ml)	Percentage unstained cells
0	Control	66
	0.1	74
	10	62
48	Control	60
	0.1	65
	10	61

most of the cells had undergone at least two divisions (see next section). Most of the stained cells were pale pink in color rather than red and probably repre-

sent cells undergoing changes in permeability as a result of trypsin digestion. A much higher percentage of unstained cells can be obtained by brushing them off the glass with a rubber policeman. However, it is impossible to completely dissociate chick fibroblasts in this way and the presence of large numbers of small clumps makes an accurate viability count out of the question.

Incorporation of Thymidine-H³ into Interphase and Mitotic Chick Embryo Cells

Information on both the proportion of cells in DNA synthesis and the mean generation time of the cells can be obtained from thymidine-H³ incorporation data. In this experiment the cells were exposed to thymidine-H³ for 20 minutes. The radioactive medium was then replaced with non-radioactive medium containing excess thymidine as described in the methods. Cover slips were removed at appropriate intervals and immersed in growth medium containing 0.06 μg/ml colchicine for 1 hour to collect mitotic figures before fixing and treating further. The data are summarized in Fig. 3.

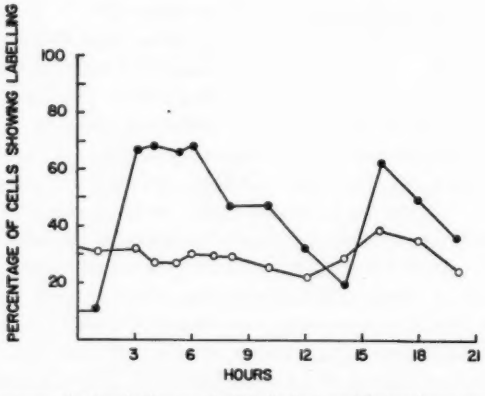

FIG. 3. Percentage of mitotic figures (solid circles) and interphase nuclei (open circles) showing labelling at various times after exposure of the cells to thymidine-H³ for 20 minutes.

Thirty-two per cent of the interphase cells was labelled during the 20-minute exposure to thymidine-H³ and this proportion of labelled cells remained fairly constant throughout the experiment indicating that about one third of the cells was in DNA synthesis during the 20-minute exposure and that these radioactive cells were as capable of dividing as those which were not labelled. Grains were not found over many of the mitotic figures until about 3 hours after removal of thymidine-H³. The percentage of labelled mitoses remained high (about 70%) for another 3 hours and then fell and rose again about 16 hours after thymidine-H³ removal. From these measurements it can be estimated that on the average about 3 hours elapse between the end of DNA replication and the metaphase stage of mitosis. This figure was very similar to that arrived at by Taylor (6) for hamster cells. The mean generation time for chick embryo

fibroblasts was about 12 hours (the distance between the two peaks of the curve in Fig. 3), which was again similar to the 14 hours estimated by Taylor for hamster cells. The length of time the cells spent in DNA replication (mean generation time × % cells labelled after 20 minutes) was about 4 hours compared with 6 hours for hamster cells. All these cell growth constants were, therefore, of the same order as found by another author for a different species of cell.

Incorporation of Uridine-H³ into Nuclear RNA

Figure 4 shows the relationship between the time of exposure to uridine-H³ and the mean number of grains over the nucleus and nucleolus. For the 20-minute uptake period the relationship was virtually linear and almost all

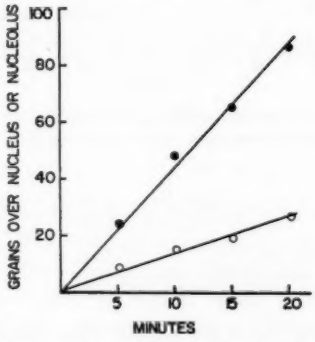

FIG. 4. Number of grains over the whole nucleus (solid circles) and nucleolus (open circles) of chick fibroblasts at various times during uptake of uridine-H³.

the radioactivity could be removed by 2 hours' treatment with ribonuclease. Very little incorporation of uridine-H³ into DNA was therefore found during the uptake period. No radioactivity above background was detected in the cytoplasm even at 20 minutes. This confirms the observation that the primary synthetic site for RNA is the nucleus and that time must elapse before labelled RNA appears in the cytoplasm.

Transfer of Labelled RNA from Nucleus to Cytoplasm

Although little or no tritium incorporation into DNA occurred during the uptake experiment with uridine-H³ there was a considerable amount detectable during turnover from nucleus to cytoplasm in those cells which were in DNA synthesis. Accordingly in the following experiment only the 68% of cells was included which showed the lowest grain counts, since 32% was shown to be in DNA synthesis by the thymidine experiment. The results of a typical transfer experiment are shown in Fig. 5.

In this experiment, the cells were exposed to uridine-H³ for 20 minutes before it was removed and the soluble precursors diluted out with unlabelled uridine and cytidine. Cover slips were then removed at required intervals and treated as outlined in the methods.

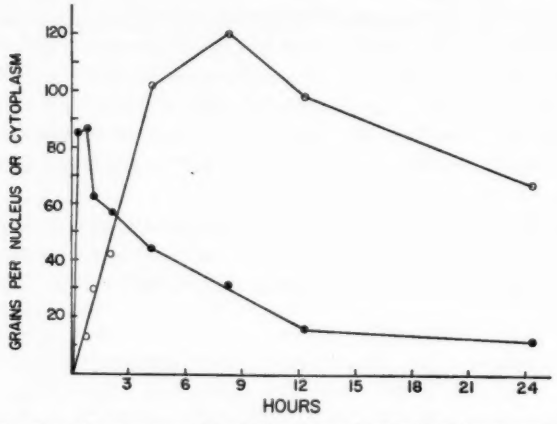

FIG. 5. Number of grains over the whole nucleus (solid circles) and over the cytoplasm (open circles) of chick fibroblasts at various times during and after 20 minutes' exposure to uridine-H^3.

During the 30 minutes after removal of labelled uridine, the nucleus showed a consistent small rise in grain counts probably due to soluble precursors which were not diluted out by unlabelled uridine and cytidine. The cytoplasm also showed labelling at this time. After 30 minutes, the level of labelling fell rapidly in the nucleus and rose rapidly in the cytoplasm. The half life of labelled RNA in the nucleus was about 4 hours. The increase in cytoplasmic labelling was much greater than it should have been and this observation agree with those of Harris on rabbit fibroblasts (5) and of Taylor on hamster cells (6). This apparent increase may be due to (*a*) soluble precursors which became incorporated into the RNA after removal of the labelled precursor, or (*b*) the geometry of the cells which were much thicker in the nuclear region than at the outer parts of the cytoplasm and hence absorbed more radiation internally. After about 8 hours, label began to leave the cytoplasm as well as the nucleus, showing that, in these cells, a slow turnover of cytoplasmic RNA did seem to occur.

The Relative Importance of Nucleus and Nucleolus in RNA Synthesis

Chick fibroblast nuclei show one or more prominent nucleoli and advantage was taken of this to make differential counts of grains over the nucleoli and the remainder of the nucleus. Table II shows the ratios of the counts at various times during the uptake of uridine-H^3 and transfer of RNA from nucleus to cytoplasm. It can be seen that the ratios vary little throughout the experiment, indicating that RNA incorporation of uridine-H^3 goes on at about the same rate at both sites.

RNA Synthesis during Mitosis

In this experiment the cells were exposed to tritium-labelled uridine for 10 minutes, cover slips being removed for autoradiography at 5 minutes and

TABLE II

Ratio of grains over nucleolus: grains over whole nucleus during uptake of uridine-H^3 into the nucleus and transfer to the cytoplasm

Time after addition of uridine-H^3 to medium	No. of grains over nucleolus / No. of grains over nucleus
5 minutes	0.35
10 minutes	0.29
15 minutes	0.28
20 minutes	0.28
20 minutes + 10 minutes*	0.34
20 minutes + 1 hour	0.35
20 minutes + 2 hours	0.29
20 minutes + 8 hours	0.31
20 minutes + 24 hours	0.34

*Additional time spent in non-radioactive medium containing excess uridine and cytidine.

10 minutes. The labelled uridine was then removed and the cells allowed to go on growing in unlabelled medium for a further 40 minutes, cover slips being removed for autoradiography at 10, 20, and 40 minutes after removal of the labelled uridine. After film exposure and development, the slides were examined for labelled mitotic figures. Between 20 and 30 cells in mitosis were counted at each stage of mitosis listed in Table III. Only cells showing more than twice the background number of grains were considered to be labelled.

TABLE III

Percentage of cells in mitosis showing labelled RNA at various times during and after exposure to uridine-H^3

Stage of mitosis*	5 min	10 min†	10 min +10 min	10 min +20 min	10 min +40 min
Early prophase	80	87	97	100	100
Late prophase	27	37	57	90	100
Metaphase	7	3	17	53	90
Anaphase	3	9	7	50	100
Early telophase	20	32	33	47	100
Late telophase	43	50	52	72	90

*Differentiation between early and late prophase and telophase depended on the extent of chromosome contraction.
†Uridine-H^3 removed at 10 minutes. Last three columns represent additional time lapse in non-radioactive medium containing excess uridine and cytidine.

From Table III it can be seen that after 5 minutes' exposure to uridine-H^3 most early prophases and some late telophases were labelled. Most of the cells in other stages of mitosis failed to incorporate uridine-H^3 into RNA. The small percentage of cells recorded as labelled in these stages may simply be due to the chance occurrence of background grains over the cells. The number of grains over such cells was small and usually only one or two more than twice the background number. After another 5-minute exposure the picture was very similar except that the proportion of labelled cells in late prophase and early telophase rose slightly. Ten minutes later an increase in metaphase

labelling was apparent 30 minutes after the start of the experiment and by 50 minutes most stages of mitosis showed uridine-H^3 incorporation.

Discussion

The main purpose of these experiments was to obtain basic data for various RNA and DNA synthetic properties of normal chick fibroblasts growing in culture. Since these cells are only second generation in culture and are isolated by simple trypsinization of chick embryo tissue, they cannot be termed pure cultures of homogeneous cell lines. On the other hand, they do not suffer from the disadvantage of long-term selection for in vitro growth conditions and are easily converted by virus infection into malignant sarcoma cells. It was important to know, therefore, that reproducible results could be obtained with such cells for a variety of measurements using tritium-labelled RNA and DNA precursors and the stripping film autoradiographic technique.

The results confirm those found for a variety of species of cells, by a number of workers over the past year or two (2, 3, 4, 5, 6, 7), that the primary sites of RNA synthesis are the nucleolus and nucleus. They also support the contention that nucleolar and nuclear RNA synthesis are separate events both contributing RNA to the cytoplasm. Uridine-H^3 was taken up by both sites beginning at zero time and at constant rates. Although the nucleolus concentrated relatively more radioactivity than the rest of the nucleus (about one third of the grains was found over the nucleolus), the ratio of radioactivity in the nucleolus to that in the nucleus remained constant over the whole experiment.

The chick fibroblasts used in these experiments showed a slight increase in grain counts over the nucleus during the first 30 minutes after removal of labelled uridine and its dilution in the cell with 25 times the concentration of non-labelled uridine and cytidine. This could be due to persistence of a small pool of radioactive precursors or to a migration of RNA to the surface of the nucleus from the interior where internal absorption would be expected to mask some of the radioactivity. Since the ratio of grains over the nucleolus to grains over the nucleus remained constant before and after removal of labelled uridine it is more likely that the increased grain counts were a result of incomplete dilution of precursors.

Labelled RNA began to appear in the cytoplasm almost immediately after removal of labelled uridine from the medium and rose rapidly as that in the nucleus was lost. As Taylor (6) has pointed out, truly quantitative measurements are impossible within a very short period after transfer begins because of the changing geometry of the cells resulting in less internal absorption as the labelled RNA moves to the periphery of the cell. The marked increase in grain counts during transfer was to be expected, therefore, and measurements are only approximate. However, a turnover of RNA from the cytoplasm to the medium appeared to occur after about 8 hours.

With regard to the thymidine experiments, the objection may be raised that the concentration of thymidine-H^3 in the medium was high, especially since

it was shown that the presence of tritium affected DNA synthesis in *vicia faba* roots (13) and that HeLa cell viability was reduced by concentrations of thymidine-H^3 greater than 0.1 μc/ml (14). However, these effects occurred when the tritium was allowed to build up in the cell over many hours. In the experiments reported here, the maximum time for incorporation to proceed was 20 minutes. Moreover, no evidence of decreased cell viability was observed for the duration of the experiments as compared with non-radioactive control preparations.

Approximately one third of the cells was capable of synthesizing DNA at any one time. This information was used to correct for uridine incorporation into DNA in the RNA transfer experiments. The combined information from the thymidine-H^3 and uridine-H^3 uptake during mitosis experiments can be used to make some attempt at timing the sequence of certain events in the chick fibroblast growing in vitro. The mean generation time (time elapsing between two mitoses) of a cell was approximately 12 hours. Of this about 4 hours was spent in DNA replication. Some 3 hours after the end of DNA replication the cell was in metaphase. RNA synthesis virtually stopped during contraction of the chromosomes in the mitotic cycle (Table III). The duration of these phases was about 30 minutes for chick fibroblasts under our conditions. Since it took about 30 minutes for the cell to reach metaphase from early prophase (Table III) about 2.5 hours elapsed between the end of DNA replication and the end of early prophase. The mitotic cycle then took another 30–40 minutes to complete, leaving between 4.5 and 5 hours before DNA replication began again. These times are, of course, approximate and are very similar to those calculated for hamster cell strains maintained several months in culture by Taylor (6).

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ACETOACETATE AND β-HYDROXY-β-METHYL GLUTARYL COENZYME A METABOLISM IN NORMAL AND KETOTIC GUINEA PIGS[1]

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Abstract

Acetoacetate metabolism was studied in normal and ketotic guinea pigs. Labelled acetoacetate was readily utilized by ketotic guinea pigs for oxidation and sterol synthesis. The results do not support the recent suggestion that hyperketonemia results from impaired ketone body utilization.

The results of some in vivo and in vitro experiments indicate that although the cleavage of β-hydroxy-β-methylglutaryl CoA (HMG-CoA) is probably the major source of acetoacetate, some acetoacetate synthesis, at least in vitro, may proceed via a pathway that does not involve HMG-CoA.

There was no correlation between the degree of activity of the HMG-CoA cleavage enzyme and sterol synthesis.

Introduction

Recent studies have provided evidence that the ketosis of diabetes and fasting is primarily a manifestation of increased ketogenesis (1–3) and the supposition that ketone bodies accumulate as a result of decreased acetyl-CoA[2] utilization (4, 5) does not appear to be supported by experimental findings (3). Although fatty acid synthesis is decreased in the ketotic state (6), acetate oxidation is not impaired (3, 7) and recent work indicates that acetyl-CoA probably does not accumulate in the diabetic liver (1). There is no general agreement as to whether or not acetoacetate utilization is impaired in the ketotic animal; however, if such impairment exists, it is unlikely that it contributes significantly to the increased ketone body concentration present in the ketotic animal. Earlier work indicated that acetoacetate (8) and DL-β-hydroxybutyrate (9) utilization in vivo is not impaired in diabetic ketotic animals. More recently, however, Beatty *et al.* (10) reported decreased acetoacetate utilization by diaphragm and muscle fibers of diabetic rats and Scow and Chernick (11) observed decreased acetoacetate and D-β-hydroxybutyrate utilization in pancreatectomized rats depleted of tissue lipids.

Acetoacetate synthesis has been studied in considerable detail since Bachhawat *et al.* (12) found that the HMG-CoA cleavage enzyme catalyzes the reaction

$$\text{HMG-CoA} \rightarrow \text{acetoacetate} + \text{acetyl CoA}. \qquad [1]$$

Later, Rudney (13) demonstrated the occurrence of an HMG-CoA condensing

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[2]The following abbreviations are used: HMG, β-hydroxy-β-methylglutaric acid; CoA, coenzyme A; DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate; AcAc, acetoacetate.

enzyme which catalyzes the reaction in yeast and liver extracts

$$\text{acetoacetyl-CoA} + \text{acetyl-CoA} + H_2O \rightarrow \text{HMG-CoA} + \text{CoASH}. \quad [2]$$

Lynen *et al.* (14) studied acetoacetate synthesis in extracts prepared from acetone powders of beef liver and obtained evidence that it proceeds via HMG-CoA through the combined effects of the HMG-CoA condensing and cleavage enzymes.

Previous work from this laboratory (3) indicated that the conversion in vivo of labelled acetate to CO_2 and sterol is not impaired in the fasted ketotic state. The present report deals with acetoacetate utilization in normal and ketotic guinea pigs and the incorporation of tracer carbon from acetate-1C^{14} into C-1 and C-3 of acetoacetate. Some aspects of HMG-CoA metabolism in the normal and ketotic state were studied. HMG-CoA cleavage enzyme activity and its relationship to sterol synthesis was investigated and evidence was found that acetoacetate synthesis in vitro may proceed by a pathway(s) other than HMG-CoA cleavage.

Materials

Acetoacetate was prepared by hydrolyzing the ethyl ester by the method of Ljunggren (15), ethanol was removed by vacuum distillation, and the sodium salt stored at −18°. The acetoacetate solution was adjusted to pH 7 before use. Ethyl acetoacetate-3-C^{14} (New England Nuclear Corporation) was also hydrolyzed before use.

The anhydride of β-hydroxy-β-methylglutaric acid (California Corporation for Biochemical Research) was prepared as described by Hilz *et al.* (16) and recrystallized twice from dry benzene in the form of fine white needles (m.p. 101–102°). The anhydride was added to reduced CoA (Pabst) and allowed to react until the sodium nitroprusside test for free sulphydryl (17) was negative. DL-HMG-CoA was assayed as the ferric chloride complex of the hydroxamic acid derivative (18, 19). HMG-3-C^{14} (Tracerlab) was diluted with unlabelled HMG prior to preparing the CoA derivative.

Methods

Adult guinea pigs weighing 895 ± 21.9 g were used for the in vivo experiments. Experimental ketosis was produced by starving guinea pigs for 48 hours. Non-pregnant fed (N.P.F.) rather than pregnant fed (P.F.) guinea pigs were used as control animals in the acetoacetate utilization studies because previous studies (3) had indicated that the N.P.F. group oxidized acetate-1-C^{14} slightly faster than the P.F. group (probably because of less tracer dilution). Similarly, it is possible that N.P.F. guinea pigs may oxidize labelled acetoacetate at a slightly higher rate than P.F. guinea pigs, since acetoacetate is converted to acetate prior to oxidation. Therefore, differences in acetoacetate oxidation should be more easily detectable by comparing ketotic with N.P.F. rather than P.F. animals.

Acetoacetate-3-C^{14} (5.6 mg, 40 μc) was injected intraperitoneally into guinea pigs and the expired CO_2 was trapped in alkali for 3 hours after dosing. The $C^{14}O_2$ was counted as $BaCO_3$ and corrections were made for self-absorption. The experimental data are presented in groups because the conditions of experiment were uniform within, but not between, groups.

Ketonemia was induced in normal guinea pigs by intraperitoneal injection of 0.61 *M* acetoacetate (pH 7). A single injection of 622 mg acetoacetate to a 1000-g animal resulted in 24 mg acetoacetate/100 ml blood in 30 minutes but this decreased to 0.5 mg acetoacetate/100 ml blood after 3 hours. If acetoacetate was injected at 30-minute intervals, the blood acetoacetate concentration remained constant for 3 hours. The animals represented in group 3 of Table II were given an initial injection of 435 mg acetoacetate. After 30 minutes they were anesthesized with ether, 1 ml blood was removed by cardiac puncture for acetoacetate analysis, and thin polyethylene tubing (1.06 mm O.D.) was inserted into the peritoneal cavity and taped in position. Labelled acetoacetate was injected and the animals were placed in the metabolism cage. The polyethylene tubing was passed to the outside of the cage through a rubber stopper. Blood acetoacetate concentrations were maintained by injecting 126 mg acetoacetate through the tubing at 30-minute intervals throughout the experiment. This procedure caused no apparent discomfort to the animals. After 3 hours, the animals were anesthesized, killed, and the liver was removed for sterol analysis.

C^{14} incorporation into liver sterol was determined as before (3). Acetoacetate was assayed by the slightly modified (20) method of Walker (21).

Sodium acetate-1-C^{14} (100 μc) was injected intraperitoneally into ketotic guinea pigs in the experiments investigating 1-C^{14} and 3-C^{14} activity of acetoacetate. Blood samples were taken 1 and 2 hours after acetate injection and deproteinized. The acetoacetate in the protein-free supernatant was decarboxylated with aniline citrate (22) or diazotized *p*-nitroaniline (21) in sealed Warburg flasks and the $C^{14}O_2$ counted as $BaCO_3$. Corrections were made for self-absorption. The results obtained with the two methods usually agreed closely; however, with two filtrates, greater C^{14} activity was observed with aniline decarboxylation. Subsequently, acetoacetate-1-C^{14} radioactivity was determined by incubating the trichloracetic acid filtrates for 30 minutes (20°) with diazotized *p*-nitroaniline. The radioactivity in C-3 of acetoacetate was measured as the formazan derivative (21) which was extracted with ethyl acetate, washed, dried in vacuum, and plated on glass cover slips. Quantitative recovery of the C^{14} activity in C-3 of acetoacetate was obtained with this method.

The in vitro studies (Figs. 1 and 2, Tables V, VI, and VII) were carried out with whole liver homogenates prepared as described previously (23). Liver was homogenized in 0.04 *M* potassium phosphate, 0.03 *M* nicotinamide, 0.007 *M* magnesium chloride, and 0.126 *M* sucrose (24) (pH 7.5), final volume as indicated. DPN (5 μmoles) and ATP (2 μmoles) were added, except when HMG-CoA was added to the incubate.

Results

The CO_2 data are divided into three groups, each group consisting of experimental and control animals. The results of group 1 (Table I) indicate that animals in the early stage of ketosis show an initial lag in acetoacetate-3-C^{14} oxidation (significant from 40 to 100 minutes) but that total $C^{14}O_2$ expired from 120 to 180 minutes did not differ significantly between the two groups. However, animals of group 2 with advanced ketosis showed decreased acetoacetate-3-C^{14} oxidation throughout the 180 minutes, indicating that ketone body oxidation may be impaired in the latter stages of fasting ketosis.

Temporary ketonemia was induced in animals 1 and 2 of group 3 (Table II) by intraperitoneal injection of acetoacetate as described above. The blood acetoacetate concentration for animals 1 and 2 remained approximately 12 mg per 100 ml throughout the experiment. The results of this experiment indicate that the lag in acetoacetate oxidation present in ketotic animals can be explained, at least in part, on the basis of an increased acetoacetate pool size.

The in vivo incorporation of acetoacetate-3-C^{14} into digitonide precipitable sterols (Table III) did not differ significantly between the control and ketotic groups. Since it was previously observed that acetate incorporation into liver sterols was accelerated in fasting ketosis of guinea pigs (3) it is not clear why this increase was not observed with acetoacetate, an intermediate of sterol synthesis. It is possible that in the ketotic state acetoacetate activation to its CoA derivative may be a rate-limiting step.

The results in Tables IV and V show that the C-1 to C-3 labelling ratio of acetoacetate synthesized in vivo was different from that synthesized in vitro. Acetoacetate synthesized in vivo from acetate-1-C^{14} was preferentially labelled in the carboxyl carbon by ratios of approximately 10 to 1. In contrast, the ratio of C^{14} radioactivity in the carboxyl and carbonyl carbons of acetoacetate synthesized in vitro was close to unity. Symmetrically labelled acetoacetate could arise either through deacylase action on acetoacetyl-CoA or possibly through cleavage of HMG-CoA, provided that only negligible amounts of unlabelled acetoacetyl-CoA originated from fatty acid oxidation during the incubation period and that most of the HMG-CoA was synthesized from acetate.

In agreement with the proposed mechanism of action of HMG-CoA cleavage enzyme (14), acetoacetate synthesized from DL-HMG-CoA-3C^{14} was almost exclusively labelled in the carbonyl carbon. The small amount of radioactivity in the number 1 carbon could have resulted from non-enzymatic degradation of labelled acetoacetate to acetate with subsequent recycling.

The amount of acetoacetate synthesized from DL-HMG-CoA (5.4 μmoles, final volume 2 ml, Fig. 1) was proportional to the amount of crude liver preparation over a limited range. The lag in the curve at lowest enzyme concentration was noted repeatedly and may represent a dilution effect. Acetoacetate synthesis was linear with time when experimental conditions were as described.

The mechanism of acetoacetate synthesis in vitro was investigated (Fig. 2)

TABLE I

Tracer incorporated into CO_2 during 3-hour period following intraperitoneal injection of acetoacetate-3-C^{14}

Time (minutes)	Total $C^{14}O_2$ expired group 1* Ketotic group (4)‡ (c.p.m. $\times 10^{-2}$)	Total $C^{14}O_2$ expired group 1* Control group (3)‡‖ (c.p.m. $\times 10^{-2}$)	*P* values	Time (minutes)	Total $C^{14}O_2$ expired group 2† Ketotic group (2)‡ (c.p.m. $\times 10^{-2}$)	Total $C^{14}O_2$ expired group 2† Control group (2)‡‖ (c.p.m. $\times 10^{-2}$)
20	42 ± 6§	94 ± 22§	> .05	20	86	142
40	155 ± 23	349 ± 71	< .05	40	112	413
60	445 ± 50	750 ± 106	< .05	60	261	878
80	921 ± 103	1386 ± 109	< .01	80	490	1486
100	1417 ± 182	1919 ± 58	< .05	100	728	2140
120	1847 ± 222	2325 ± 88	> .05	120	1012	2739
140	2333 ± 181	2687 ± 115	> .05	140	1321	2913
160	2599 ± 244	2852 ± 162	> .05	160	1512	3204
180	2691 ± 244	3099 ± 296	> .05	180	1966	3809

*Group 1: 4.5, 6.5, 13.7, and 17.6 mg acetoacetate/100 ml blood.
†Group 2: 19.8 and 21.6 mg acetoacetate/100 ml blood.
‡Numbers in parentheses indicate number of animals used.
§Standard error of mean.
‖Control animals (group 1 and 2): <0.2 mg acetoacetate/100 ml blood.

TABLE II

Tracer incorporated into CO_2 during 3-hour period following intraperitoneal injection of acetoacetate-3-C^{14}

Time (minutes)	Total $C^{14}O_2$ expired group 3		
	Induced ketonemia		
	Animal No. 1* (c.p.m. $\times 10^{-2}$)	Animal No. 2† (c.p.m. $\times 10^{-2}$)	Control animal No. 3‡ (c.p.m. $\times 10^{-2}$)
20	34	63	117
40	144	261	414
60	395	600	968
80	774	1274	1740
100	1331	1788	2392
120	1731	2229	2973
140	2289	3150	3418
160	2743	3569	—
180	2988	3700	3864

*Animal No. 1: (*a*) Time, 0 minute; blood acetoacetate, 11.97 mg/100 ml.
(*b*) Time, 180 minutes; blood acetoacetate, 11.63 mg/100 ml.
†Animal No. 2: (*a*) Time, 0 minute; blood acetoacetate, 12.15 mg/100 ml.
(*b*) Time, 180 minutes; blood acetoacetate, 11.69 mg/100 ml.
‡Animal No. 3: blood acetoacetate <0.1 mg/100 ml.

TABLE III

Incorporation of C^{14} into sterols of liver following intraperitoneal injection of acetoacetate-3-C^{14}

Group	mg AcAc/100 ml blood	Sterol (mg/g tissue)	% incorporation $\times 10^3$	Sterol (c.p.m./mg)
Control (7)‡	<0.20	2.53±0.106*	14.0±4.30	60±17.7
Ketotic (11)	13.18±2.35	3.43±0.440†	12.8±3.02†	68±18.5†

*Standard error of mean.
†Does not differ significantly from other group.
‡Numbers in parentheses indicate number of animals used.

TABLE IV

C^{14} incorporation into C-1 and C-3 of acetoacetate in blood following intraperitoneal injection of acetate-1-C^{14}

Time of sampling after tracer injection (hours)	mg AcAc/100 ml blood	c.p.m./mg AcAc		$\frac{C^{14}OOH}{C^{14}O}$ ratio
		C-1	C-3	
1	14.85	120,781	9,764	12.4:1
1	19.40	94,023	7,534	12.5:1
2*	9.35	74,182	2,246	33.0:1
2	20.38	15,741	3,607	4.4:1
2	10.51	47,344	5,352	8.9:1
2	8.30	25,830	874	20.0:1
2	8.13	24,797	1,784	13.9:1
2	14.08	36,701	3,161	11.6:1

*Acetoacetate was decarboxylated with aniline citrate (22). All other samples were decarboxylated by incubating with diazotized *p*-nitroaniline (21).

TABLE V

C^{14} incorporation into C-1 and C-3 of acetoacetate by liver homogenates

Experiment No.	Substrate	C-1 (total c.p.m.)	C-3 (total c.p.m.)	$\frac{C^{14}OOH}{C^{14}O}$ ratio
1	Acetate-1-C^{14}	23,680	26,740	0.9:1
2	Acetate-1C^{14}	55,821	64,535	0.9:1
3	Acetate-1C^{14}	187,744	83,592	2.3:1
4	Acetate-1C^{14}	64,189	48,995	1.3:1
4*	DL-HMG-3-C^{14}CoA	535	130,777	

NOTE: Two milliliters of liver homogenate incubated in 0.044 *M* potassium phosphate, 0.03 *M* nicotinamide, 0.007 *M* $MgCl_2$, 0.126 *M* sucrose. Additions: 5 μmoles DPN, 2 μmoles ATP, 0.7 μmole acetate-1-C^{14}. Final volume 2.6 ml, pH 7.5. Incubation 60 minutes, gas phase O_2.
*0.3 ml homogenate, 2.7 μmoles DL-HMG-CoA-3C^{14}, pH 7.5, final volume 2.0 ml. Incubated 30 minutes in O_2. ATP and DPN omitted.

by incubating acetate-1C^{14} with 0.3 ml liver homogenate in the presence of increasing concentrations of HMG-CoA. If acetoacetate were synthesized by a single pathway, i.e. via HMG-CoA as intermediate, increasing the HMG-CoA concentration of the incubates should result in a progressive decrease of acetate incorporated into acetoacetate.

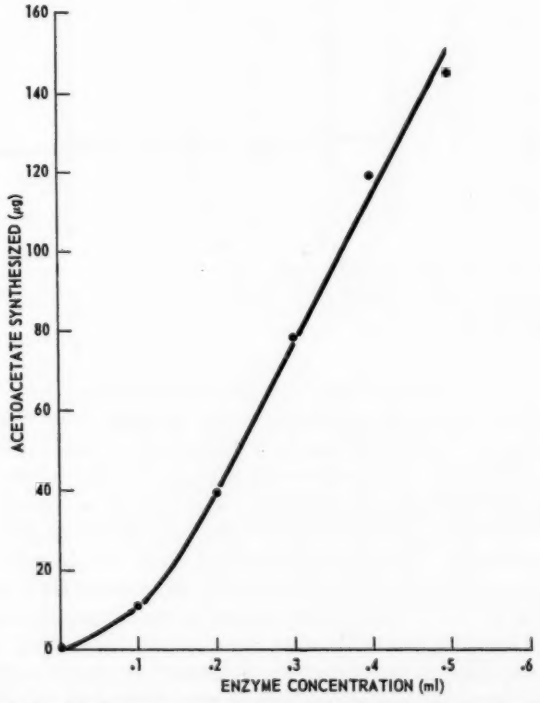

FIG. 1. Relationship between acetoacetate synthesis and enzyme concentration when incubated 30 minutes at 37° in O_2 with 5.4 μmoles DL-HMG-CoA added.

An initial decrease in acetate-1C^{14} incorporation would result from a dilution of the radioactive acetate by acetyl-CoA resulting from HMG-CoA cleavage. The results illustrated in Fig.2 show that after an initial decrease in the radioactivity of C-1 and C-3 of acetoacetate, further increases in HMG-CoA concentration did not decrease acetoacetate radioactivity. If the assumption is made that HMG-CoA diffuses freely through the mitochondrial membrane and that actual dilution of the cleavage enzyme accessible HMG-CoA pool was achieved, then these results indicate that at least two pathways are operative in the in vitro synthesis of acetoacetate, one of which does not involve HMG-CoA.

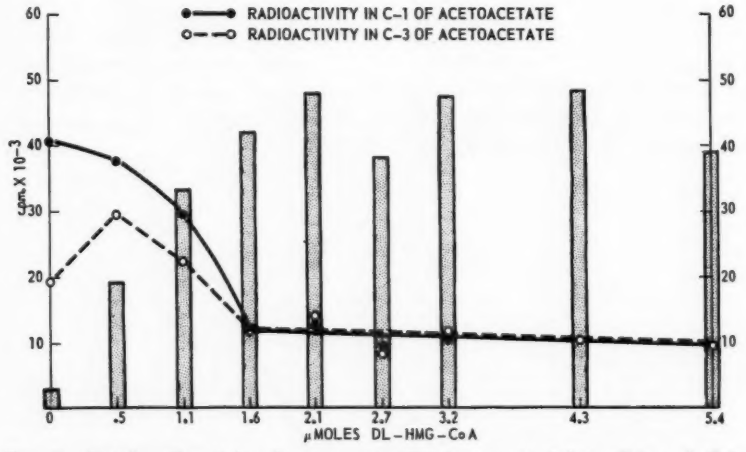

FIG. 2. Total radioactivity from acetate-1C^{14} incorporated into C-1 and C-3 of acetoacetate by 0.3 ml liver homogenate incubated for 30 minutes in the presence of increasing concentrations of DL-HMG-CoA. Vertical bars indicate total acetoacetate (μg) synthesized by each incubate.

Results in Table VI show HMG-CoA cleavage enzyme activity in normal and ketotic animals as related to sterol synthesis. Aliquots from the same homogenate were used for both sterol and acetoacetate synthesis. Liver homogenate (0.3 ml, 9.7 mg protein, final volume 2.0 ml) was incubated with 5.4 μmoles DL-HMG-CoA for acetoacetate synthesis. The liver was homogenized as described previously (23) and no special attempt was made to activate the cleavage enzyme. (25). The liver preparation from the ketotic animal had greater endogenous acetoacetate synthesis (3) but lower HMG-CoA cleavage enzyme activity than the appropriate controls. In agreement with the previously observed decrease in metabolic activity of ketotic liver preparations (3, 26, 27), the conversion of mevalonic acid-2-C^{14} to sterol was also reduced. It has already been demonstrated that the failure to synthesize sterol from acetate in liver preparations from fasted animals is not related to in-

TABLE VI

The in vitro relationship of HMG-CoA cleavage enzyme activity to sterol synthesis

Status of animal	Liver homogenate incubated (ml)	Substrate added	AcAc synthesized (μg)	Cholesterol synthesized (total c.p.m.)
1. Fed (adult)	0.3	—	3.12	—
	0.3	DL-HMG-CoA	56.24	—
	2.0	Mevalonate-2-C^{14}	—	38,349
2. Fasted ketotic	0.3	—	13.68	—
	0.3	DL-HMG-CoA	42.24	—
	2.0	Mevalonate-2-C^{14}	—	16,917
3. Fed (young)	0.3	—	7.36	—
	0.3	DL-HMG-CoA	62.40	—
	2.0	Acetate-1-C^{14}	—	37,938
4. Fed (young)	0.3	—	7.36	—
	0.3	DL-HMG-CoA	51.04	—
	2.0	Acetate-1C^{14}	—	18,790
5. Fed (young)	0.3	DL-HMG-CoA	83.92	—
	2.0	Acetate-1C^{14}	—	46,875
6. Fed (young)	0.3	DL-HMG-CoA	73.04	—
	2.0	Acetate-1-C^{14}	—	53,479

NOTE: Incubation procedure as in Table V. Homogenates assayed for acetoacetate incubated 30 minutes. ATP and DPN omitted. Homogenates assayed for sterol synthesis incubated 90 minutes with 5 μmoles DPN and 2 μmoles ATP added.

creased HMG-CoA cleavage enzyme activity (25). The present results in support of this finding show that rate of sterol synthesis is not inversely proportional to HMG-CoA cleavage enzyme activity.

TABLE VII

The in vitro incorporation of labelled substrates into cholesterol by guinea pig liver supernatant fraction

Experiment No.	Substrate	Cholesterol incorporation (μmoles×10^3)
1	Acetate-1-C^{14} (0.7 μmole)	6.79
	DL-HMG-3-C^{14}-CoA (2.7 μmoles)	0.36
	DL-Mevalonate-2-C^{14} (0.6 μmole)	59.62
2	DL-HMG-3C^{14}-CoA (2.7 μmoles)	0.81
	DL-Mevalonate-2-C^{14} (0.6 μmole)	50.25

NOTE: Liver homogenate was centrifuged for 10 minutes at 10,000×g and incubated 2 hours at 37° in O_2. Incubation procedure as in Table V; 5 μmoles DPN and 2 μmoles ATP added.

The results of incubating DL-HMG-3C^{14} CoA with a 10,000×g supernatant fraction of guinea pig liver are shown in Table VII. Although the reason for the low rate of cholesterol synthesis from the added HMG-CoA is not clear since it has been shown (25) that rat liver microsomes readily reduce HMG-CoA to mevalonic acid, it may be related to the HMG-CoA cleavage enzyme activity that remained in the supernatant fraction.

Discussion

Conflicting reports have been published about the ability of the ketotic animal to utilize acetoacetate. Earlier work indicated that ketone body utilization is not impaired in ketosis (8, 9, 28) while more recent reports indicate that some impairment may exist (10, 11).

The results of the present investigation indicate that acetoacetate utilization, either for sterol synthesis or oxidation, is not grossly impaired in fasting hypoglycemic ketosis. That the initial lag in labelled acetoacetate oxidation may simply be due to a dilution effect was supported by the observation that normal animals with induced ketonemia showed a similar lag. On the other hand, acetoacetate 3-C^{14} oxidation was decreased throughout the 3-hour experimental period in two animals with advanced ketosis. The present results do not support the conclusion that hyperketonemia is the result of decreased ketone body utilization (10, 11) since increased blood acetoacetate concentrations were observed in animals that did not have impaired ability to oxidize acetoacetate. However, these results do indicate that, in the terminal stages of ketosis due to fasting, acetoacetate oxidation may be decreased to some extent.

Blood acetoacetate concentration in ketotic guinea pigs, instead of increasing progressively, is characterized by an increase followed by a decrease in the terminal stages. The present results indicate that ketosis of fasted guinea pigs is a manifestation of increased, not decreased, metabolic activity, and because of the temporary nature of this process the results are entirely in accord with the suggestion (11) that ketogenesis may be mediated through adrenal cortical secretions.

There is no complete agreement on the detailed pathway of acetoacetate synthesis. Lynen *et al.* (14), from studies with beef liver acetone powder extracts, proposed that acetoacetate synthesis occurs primarily through HMG-CoA as intermediate (reaction 1), a finding that has recently been confirmed by Caldwell and Drummond (29). However, Stern and Miller (30) and Drummond and Stern (31) inhibited HMG-CoA condensing and cleavage enzymes in rat and ox liver systems with iodoacetamide and noted that acetoacetate synthesis from acetoacetyl-CoA was not inhibited. This suggested the presence of a specific acetoacetyl-CoA deacylase catalyzing the reaction

$$\text{acetoacetyl-CoA} + H_2O \rightarrow \text{acetoacetate} + \text{CoASH}. \qquad [3]$$

The present data suggest that both pathways may be operative in vitro in the guinea pig liver system since increasing the HMG-CoA pool size resulted only in an initial decrease in acetate incorporation into acetoacetate and further HMG-CoA additions did not affect tracer incorporation into acetoacetate. The finding that liver homogenates synthesized acetoacetate from acetate-1C^{14} with C^{14}-1 to C^{14}-3 ratios of close to unity could also be consistent with acetoacetyl-CoA deacylase activity. However, acetoacetate synthesized in vivo from acetate-1C^{14} was always preferentially labelled in the carboxyl carbon. Although an explanation for asymmetric acetoacetate labelling has been suggested

(32), these results could be accounted for by unlabelled acetoacetyl-CoA accumulating as a result of incomplete beta oxidation of even-numbered fatty acids. Acetoacetyl-CoA, after combining with labelled acetyl-CoA to form HMG-CoA, would yield acetoacetate-1-C^{14} after cleavage.

The fact that guinea pig liver homogenates had more HMG-CoA cleavage enzyme activity than required to account for acetoacetate synthesis from acetate (23) indicates that reaction 1 cannot be rate-limiting. Although HMG-CoA cleavage enzyme is undoubtedly in a partly inactive state in the undisrupted liver, the fact that sterol synthesis takes place in vitro in the presence of active HMG-CoA cleavage enzyme may indicate either 'compartmentation', whereby the HMG-CoA synthesized by microsomes is converted to mevalonic acid by microsomal reductase (25), or the formation of an enzyme substrate complex unavailable to the HMG-CoA cleavage enzyme.

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NOTES

RECOVERY OF VASOPRESSIN FROM URINE AND BLOOD BY PHENOL EXTRACTION*†

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One approach to the investigation of the role played by neurohypophyseal hormones in clinical conditions has been to determine the concentration of the hormone in body fluids by extraction and assay. A number of extraction procedures have been used for recovering vasopressin (1, 2, 3) and oxytocin (1, 4) from urine and blood but some of these methods are reported to have low and variable recovery rates (10% and 50%–100%).

The present study was undertaken in an attempt to devise a new extraction method for vasopressin in urine and blood.

Methods

Extraction from Urine

A solution of phenol was prepared by adding enough water to give two phases. These were equilibrated by shaking in a separatory funnel and the phenol (lower) phase was used for extraction.

Fresh human urine was adjusted to pH 5.0 with glacial acetic acid, concentrated in vacuo at 50° C to 20% of its original volume, and transferred to a separatory funnel. It was then extracted 3 times with an equal volume of phenol. The phenolic extracts were separated, pooled, and mixed with 3 volumes of diethyl ether. This mixture was then extracted 3 times with a total volume of water equal to the volume of concentrated urine. The aqueous extracts were pooled and taken to dryness *in vacuo* at 50° C. The dry residue was washed with absolute ethanol, again dried, and made up to 5% of the original urine volume for bio-assay.

Extraction from Blood

Blood was obtained from heparinized rats under light ether anesthesia, it was centrifuged, and the plasma removed. Plasma proteins were precipitated by adding 1.0 cc of phenol and 0.5 cc of glacial acetic acid per 5.0 cc of plasma. The mixture was centrifuged and the clear supernatant was extracted with phenol as described above for urine. For bio-assay, the plasma extract was made up to a volume equal to the volume of whole blood extracted.

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Bio-assay Procedure

Both urine and blood extracts were assayed by the Burn technique (5) as modified by Jessup and Taylor (6). Injections, 0.5 cc per 100 g body weight, were given subcutaneously to hydrated rats and the criterion of response was the time taken to excrete 50% of the urine. Total urine excretion was considered to have occurred when more than 75% of the water load was excreted and the rate of excretion was reduced to less than 3.0 ml per hour.

Results

Percentage Recovery

Pitressin (Parke-Davis) was added to urine and blood before extraction and equivalent concentrations of vasopressin were added to extracts of both fluids after extraction. The concentrations of Pitressin ranged from 2.0 milliunits to 20.0 milliunits per 100 cc of urine and from 0.5 milliunit to 4.0 milliunits per 1.0 cc of blood or 0.4 milliunit to 4.0 milliunits per 1.0 cc of urine extract and 0.5 milliunit to 4.0 milliunits per 1.0 cc of plasma extract.

Simultaneous assays of these various extracts were carried out and the results are presented in Table I. Percentage recovery for the method was determined from the difference in activity of the extracted and non-extracted hormone. The results indicate that the phenol extraction method recovers 85% to 90% of the hormone from urine and 70% to 80% from plasma.

TABLE I

Recovery of pitressin from urine and blood

Pitressin, milliunits per:		ADH equivalent in extracts, milliunits		% recovery	
100 cc urine	1.0 cc blood	Urine	Blood	Urine	Blood
2.0	0.5	1.7	0.40	85	80
6.0	1.0	5.3	0.75	88	76
10.0	2.0	8.7	1.40	87	70
20.0	3.0	18.0	2.10	90	70
—	4.0	—	2.80	—	70

Recovery of Endogenous ADH

To determine if the method would recover the endogenously secreted hormone, the urine from dehydrated rats was extracted. Thirty-two male rats

TABLE II

Recovery of ADH from urine of dehydrated rats

Hours of dehydration	Urine volume (32 rats), cc	Urine spec. grav.	50% excretion time (min)	ADH equivalent, milliunits/rat
24	108	1.029	98 ± 0.6* (8)†	0.8
48	72	1.053	124 ± 1.0 (8)	1.2
72	64	1.063	158 ± 1.7 (8)	7.5

*Standard error of the mean.
†Number of determinations.

weighing 170–180 g were placed in metabolism cages in groups of four rats per cage. They were deprived of food and water for 72 hours, during which time urine collections were made at 24-hour intervals.

The urine collected each day from each group was subjected to the phenol extraction method and the extracts were assayed for antidiuretic activity. The results are presented in Table II. It will be noted that increasing amounts of antidiuretic substance were recovered as the time of dehydration increased. There was an increase both in the urinary concentration and in the total amount of ADH after successive days of dehydration.

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A NOTE ON THE EFFECTS OF HYPOTHERMIA ON ENZYME ACTIVITIES IN THE RAT*

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During the course of our studies on hypothermic rats (1, 2), certain observed metabolic alterations were suggestive of altered activities of tissue enzymes. It is known that, for most enzymes, a reduction in temperature decreases activity in vitro. It is known also that, in the hypothermic animal, total metabolism, respiration, blood circulation, and the functions of organs such as the liver (3) and the kidney (4) are reduced. The present experiments were carried out to examine the effects, on certain enzyme activities in the rat, of reduced temperature per se and of previously induced hypothermia.

Methods

In five experiments, fasting, male, albino rats of the Wistar strain, weighing 200–250 grams, were used. In each experiment, hypothermia was induced in one group of 9, 10, or 12 rats by placing the animals in individual, cylindrical, wire-screen cages under crushed ice until a rectal temperature of 15° C was attained. This required a period of 30–40 minutes. A second group of animals in each experiment served as controls. In all cases, animals were sacrificed by stunning, and decapitation and enzyme activities were measured at incubation temperatures of both 37° and 15° C.

*DRML Report No. 109-10; PCC No. D50-93-10-72.

In the first experiment, glucose-6-phosphatase activity was measured in liver homogenates by the procedure of Cori and Cori (5) as modified by Willmer (6). In the second experiment, catalase activity was measured in liver homogenates by the procedure of Feinstein (7). Phosphate-activated glutaminase activity was measured in kidney homogenates, in a third experiment, by the procedure of Archibald (8) as modified by Beaton and Goodwin (9). In a fourth experiment, alkaline phosphatase activity was measured in plasma of heparinized blood taken from the neck by the method of Bodansky (10). In the final experiment, glucose utilization by muscle tissue was measured by the method of Krahl and Bornstein (11) using a 20% homogenate of gluteus maximus in saline (pH 7.45) and an incubation period of 5 minutes. Enzyme activities are expressed as follows: (*a*) glucose-6-phosphatase as μg inorganic phosphorus formed from glucose-6-phosphate per 100 mg tissue in 15 minutes; (*b*) catalase activity as meq sodium perborate destroyed per mg tissue per hour; (*c*) phosphate-activated glutaminase activity as mg ammonia formed per 100 mg tissue per hour; (*d*) alkaline phosphatase activity as mg phosphorus liberated from sodium glycerophosphate substrate by 1 ml plasma in 1 hour; (*e*) glucose utilization as μg glucose utilized in 5 minutes by 100 mg muscle tissue.

Results

The results of enzyme activity determinations are shown in Table I as the mean and standard error of the mean for each group and with statistical significance of difference between means calculated by application of Student's *t* test. It is apparent that reduction of incubation temperature to 15° C per se significantly decreased the activities of liver glucose-6-phosphatase, of kidney phosphate-activated glutaminase, of plasma alkaline phosphatase, and of glucose utilization by muscle, but not of liver catalase, in both normothermic and hypothermic rats. No enzyme activity in hypothermic rats differed significantly from that in control normothermic animals when measurements were made at an incubation temperature of 15° C. Indeed, under these experimental conditions, no glucose utilization by muscle of normothermic or hypothermic animals could be detected during 5 minutes' incubation at 15° C. When measured at 37° C, liver glucose-6-phosphatase and plasma alkaline phosphatase activities and glucose utilization by muscle were significantly lower in hypothermic rats than in normothermic controls.

Discussion

Blair *et al.* (12) have reported that in pentobarbitalized, hypothermic dogs (26–27° C rectal temperature), prolonged hypothermia for 12 hours causes an elevation in serum glutamic–oxaloacetic transaminase activity which returns to normal on rewarming. In the absence of histological evidence of cell necrosis, these workers suggest that hypothermia altered cell permeability, thus permitting transfer of this enzyme from tissues to blood.

TABLE I

Enzyme activities in control and hypothermic (15° C rectal temperature) rats as measured at incubation temperatures of 37° and 15° C (Results expressed as mean ± standard error of the mean for each group)

Expt.	Incubation temperature, °C	Control group		Hypothermic group		Probability, *P*
		37 (*a*)	15 (*b*)	37 (*c*)	15 (*d*)	
1	Liver glucose-6-phosphatase activity*	600 ± 11.2 (9)	172 ± 5.8 (9)	506 ± 11.2 (9)	162 ± 2.2 (9)	(*a*) vs. (*b*) < 0.001 (*c*) vs. (*d*) < 0.001 (*a*) vs. (*c*) < 0.01
2	Liver catalase activity*	5.75 ± 0.302 (9)	5.29 ± 0.293 (9)	5.61 ± 0.507 (9)	5.63 ± 0.653 (9)	—
3	Kidney phosphate-activated glutaminase activity*	1.29 ± 0.031 (10)	0.97 ± 0.060 (10)	1.35 ± 0.025 (10)	0.96 ± 0.032 (10)	(*a*) vs. (*b*) < 0.001 (*c*) vs. (*d*) < 0.001
4	Plasma alkaline phosphatase activity*	10.1 ± 0.98 (10)	4.9 ± 0.53 (10)	5.8 ± 0.27 (10)	3.6 ± 0.53 (10)	(*a*) vs. (*b*) < 0.001 (*c*) vs. (*d*) < 0.05 (*a*) vs. (*c*) < 0.001
5	Glucose utilization by muscle (gluteus maximus)*	34 ± 3.2 (12)	None detected (12)	14 ± 1.6 (12)	None detected (12)	(*a*) vs. (*c*) < 0.001

NOTE: ( ) indicates number of animals.
*For units of activity, see text.

In our experiments, hypothermia did not cause an increase in the activity of any enzyme measured but did cause a decrease in activities of liver glucose-6-phosphatase and of plasma alkaline phosphatase which are not explainable on the basis of a temperature effect alone. Decreased utilization of glucose by muscle of hypothermic rats could represent a decreased activity of more than one enzyme of glycolysis. The liver is a rich source of glucose-6-phosphatase, and whether the decreased activity (measured at 37° C) in the hypothermic rat is due to release of the enzyme into the blood through changes in cell permeability or to destruction or inhibition of the enzyme is not known. The decreased activity of plasma alkaline phosphatase would seem to suggest an inhibitory or destructive effect of hypothermia on this enzyme. Since blood pack cell volume is increased (1) and plasma water is decreased (2) in hypothermic rats, the lowered activity of alkaline phosphatase cannot be explained on the basis of blood dilution. In view of the marked elevation in blood inorganic phosphorus as the apparent result of catabolism of organic phosphorus compounds in hypothermic rats (1, 2), the decreased activities of these two enzymes are of considerable interest.

With regard to the relative temperature independence of liver catalase as determined here, Sizer (13) has shown that although beef catalase obeys the Arrhenius law over a wide temperature range, its energy of activation is one of the lowest reported for enzymes. Sizer's data show a Q_{10} value for beef catalase of only about 1.3 in the range 1°–40° C.

It is difficult to correlate in vitro enzyme measurements with in vivo metabolite measurement. Indeed, it cannot be assumed that altered enzyme activities as determined in vitro represent the situation in the hypothermic animal in vivo. It is clear, however, that when hypothermia is induced in a rat under these conditions, the activities of liver glucose-6-phosphatase and plasma alkaline phosphatase and glucose utilization by muscle at 37° C in vitro are significantly reduced whereas liver catalase and kidney phosphate-activated glutaminase activities are not affected by induction of hypothermia.

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ISOLATION AND IDENTIFICATION OF ADRENOSTERONE IN SALMON (ONCORHYNCHUS NERKA) PLASMA

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Adrenosterone, in a crystalline form, has been isolated from extracts of human adrenal cortex (1). It has also been identified in the perfusate obtained when blood containing added ACTH was circulated through bovine glands (2).

To our knowledge the presence of adrenosterone in peripheral plasma has not previously been reported.

In a study involving the quantitative estimation of various steroid hormones in sockeye salmon plasma (3), an unidentified ultraviolet (U.V.) absorbing spot was detected when plasma extracts prepared from postspawned male sockeye were chromatographed on paper with a heptane : 80% methanol solvent system for 16 hours. The polarity of the new substance was identical with that of adrenosterone and intermediate between 17α-hydroxyprogesterone and its 20β-dihydro-epimer, both previously isolated from salmon plasma. The Zimmermann reagent produced the violet color, typical of 17-ketosteroids, with the new steroid.

Sockeye salmon plasma was obtained from postspawned fish taken at Cultus Lake in November, 1960. The procedure for bleeding the fish and for extracting the plasma, and the chromatographic methods used, have been described elsewhere (3).

The extract from 500 ml of plasma was partitioned between 70% methanol and hexane (3). The 70% methanol fraction was then taken to dryness and chromatographed on paper using the heptane : 80% methanol system for 16 hours. The reference standard of adrenosterone and the corresponding spot on the plasma strip were detected by U.V. light (4) and had identical mobilities (R_T = 0.72 cm/hr). The plasma spot was eluted with methanol using descending chromatography. By U.V. absorption at 240 mμ (corrected) (3), and based on the recovery of adrenosterone through the procedures, there was 2.5 μg/100 ml of the steroid in the plasma. The plasma was eluted from the paper, evaporated to dryness, and subjected to sodium borohydride reduction (5). The reduction product and 11-ketotestosterone were chromatographed on paper using heptane:benzene (1:1) – 70% methanol for 3 hours. The plasma spot and the reference standard were detected with U.V. light and both had identical mobilities.

The substance from the plasma was eluted from the paper, treated with 0.1 ml of acetylating mixture, and left at room temperature for 24 hours. The reaction mixture was taken to dryness using N_2 at 40°. The acetylating mixture consisted of 5 volumes of pyridine, 4 volumes of benzene, and 1 volume of acetic anhydride. The acetylated product was chromatographed along with 11-ketotestosterone acetate using the heptane : 80% methanol solvent system for 3.75

hours and the mobilities of the steroids were identical (R_T = 4.1 cm/hr).

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A METHOD FOR THE PRESERVATION OF LEUCONOSTOC MESENTEROIDES*

S. M. LESLEY

A simple method for the preservation of cells of *Leuconostoc mesenteroides* would obviously save time and effort in the routine analysis of isotope distribution in labelled sugars. Of the published methods, that of Loewus *et al.* (1) is suitable only for large commercial preparations while lyophilization (2) was unsuccessful under our conditions. The method given here is based on freezing in 15% glycerol, as described by Quadling (3).

Actively fermenting cells, in the medium of De Moss *et al.* (4), were centrifuged and resuspended to a concentration of 100 mg per ml (wet weight) in 0.05 *M* phosphate buffer (pH 6.0) containing 15% glycerol (v/v). Individual 5-ml portions were frozen at −30° C and stored at the same temperature. When required, the cells were thawed at room temperature and incubated in 10 times the volume of De Moss medium for 30 minutes at 37° C. The cells were collected, washed once, and resuspended to the original concentration in 0.05 *M* phosphate buffer (pH 6).

During incubation, the fermentative ability of the cells was completely restored. In Fig. 1, curves 1, 2, and 3 indicate the extent of cell recovery with time of treatment. This restoration took place without any detectable increase in either cell number or mass. These cells were stable at 4° C for at least 4 hours, in contrast to freshly grown cells which rapidly lose activity at this temperature (5).

The frozen cells are stable at −30° C. In Fig. 1, curve 3 represents data obtained with frozen cells stored for either 2 days or 14 months.

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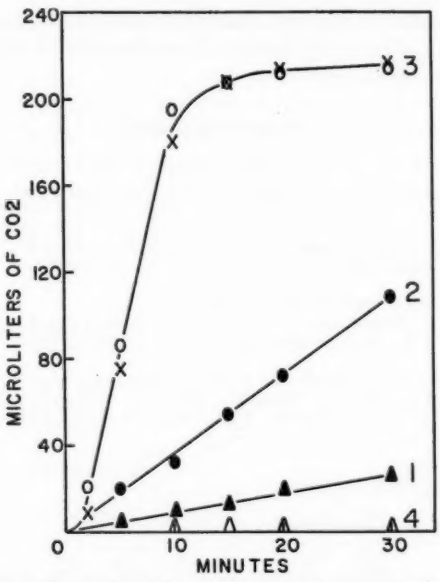

FIG. 1. Carbon dioxide produced from glucose by *L. mesenteroides* reactivated after storage in 15% glycerol at −30° C. Curves 1, 2, and 3 represent cell preparations reactivated for 2, 12, and 30 minutes, respectively. Curve 3 represents cells stored for either 2 days (○) or 14 months (×). Curve 4 is a control and represents fully reactivated, stored cells tested without glucose.

Cells stored and treated as described were used to ferment specifically labelled glucose to the usual characteristic products which were isolated by standard methods (6). The ethanol and lactic acid fractions were each oxidized directly to carbon dioxide with persulphate as described by Katz *et al.* (7). In each instance, carbon dioxide was trapped in sodium hydroxide and counted as barium carbonate. The results (Table I) indicate little cross contamination among the three products.

TABLE I

Degradation of labelled glucose with *L. mesenteroides* reactivated after storage in 15% glycerol at −30° C

	Distribution of C^{14} among fermentation products*		
Labelled substrate	Carbon dioxide	Ethanol†	Lactic acid†
D-Glucose-1-C^{14}	100	0	1.8
D-Glucose-2-C^{14}	0	100	3.7
D-Glucose-6-C^{14}	0	6.7	100

*Carbon atom of original site of labelling is assigned an arbitrary value of 100 and other carbon values compared to this.
†Oxidized to carbon dioxide and counted as described in the text.

This method of preservation of *L. mesenteroides* has proved reliable and offers the advantages of ready availability of cells combined with maximum reproducibility of biological action.

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STABILITY OF DEOXYCYTIDYLATE DEAMINASE IN VITRO*

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Previous experiments on deoxycytidylate deaminase activity in regenerating rat liver indicated that the enzyme was rapidly inactivated during incubation of tissue homogenates but retained its activity in the presence of 5 mmolar substrate (1). Similar results have been reported for thymidylate kinase (2). Both of these enzymes are involved in the synthesis of deoxyribonucleic acid and the question arose, therefore, whether the stabilizing effect of substrates on these enzymes was associated with the control of deoxyribonucleic acid synthesis in growing cells. The stability of deoxycytidylate deaminase has now been studied in more detail.

During incubation of intact isolated lobes from regenerating rat liver at 37° C for 60 minutes, deoxycytidylate deaminase activity decreased to approximately 35% of its initial value. Adenosine deaminase, thymidine phosphorylase, uridine phosphorylase, and neutral pyrophosphatase, another labile enzyme (3), were also assayed in the same liver preparations but were found to retain 90–105% of their original activity after incubation for 60 minutes.

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Seven other enzymes in liver have also been reported to retain 80% or more of their original activity under similar conditions (4).

Protection of deoxycytidylate deaminase against inactivation in vitro is illustrated in Table I. In these experiments, the supernatant fraction from a homogenate of rat thymus in 9 volumes 0.02 *M* EDTA was incubated for

TABLE I

Stabilization of deoxycytidylate deaminase in vitro

Protective compound	Michaelis constant (mmolar)	Incubation for 30 minutes at 37° C	
		Concentration (mmolar)	% initial deaminase activity remaining
None	—	—	3
Cytosine deoxyriboside-5′-phosphate	1.4	5	98
		2	70
		1	41
		0.3	10
Cytosine deoxyriboside-5′-triphosphate	—	1	85
		0.3	38
Thymine deoxyriboside-5′-phosphate	0.2	1	95
		0.3	58
		0.1	17
Adenine deoxyriboside-5′-phosphate	0.8	1	70
		0.3	30
Guanine deoxyriboside-5′-phosphate	0.07	1	100
		0.3	98
		0.1	73
		0.03	38
Guanine deoxyriboside-5′-triphosphate	0.4	1	95
		0.3	59
		0.1	20

30 minutes at pH 7.3–7.4 and 37° C in the presence or absence of protective compounds. The solutions were immediately assayed for deaminase activity in the usual manner (1) and their activities compared with those of control solutions which had been held at 0° C. EDTA was included in the enzyme preparations to prevent inactivation by contaminating metal ions and to minimize the activity of hydrolytic enzymes such as magnesium-activated phosphatases.

Among the compounds tested as protective agents, the following were found to be almost ineffective in 1 mmolar concentrations: thymine, cytosine, thymine deoxyriboside, cytosine deoxyriboside, cytosine riboside-5′-phosphate, adenine riboside-5′-phosphate, adenine riboside-5′-triphosphate, and adenine deoxyriboside-5′-triphosphate. The most effective protection was provided by compounds which partially inhibited the deaminase activity of normal tissue extracts. Michaelis constants for the enzyme–substrate complex and for the enzyme–inhibitor complex in absence of substrate were therefore estimated for fresh thymus extracts by standard methods (5) in the presence of 0.01 *M*

EDTA, 0.08 *M* Tris buffer, and 0.06 *M* NaF at pH 8.0 and 37° C (cf. 1). The results showed that the relative efficiency of the deoxyribonucleotides as protective agents was closely correlated with their affinities for the enzyme active center, either as a substrate (cytosine deoxyriboside-5′-phosphate) or as competitive inhibitors (adenine, thymine, and guanine deoxyriboside-5′-phosphates) of the enzyme (Table I).

It appears, therefore, that deoxycytidylate deaminase in tissue extracts is stabilized in vitro by deoxyribonucleotides which have a high affinity for the enzyme. We might speculate that the same compounds could stabilize the deaminase in vivo and that the changes in deaminase concentration in liver following partial hepatectomy and X irradiation (1) are determined by the changes in deoxyribonucleotide concentration (6). However, the total concentration of adenine and guanine deoxyribonucleotides in rat tissues is extremely low, probably less than 0.01 mmole/kg tissue (6–8). The total concentration of pyrimidine deoxyribonucleotides is measurable, e.g., approximately 0.09 mmole/kg regenerating liver and 0.02 mmole/kg normal liver (9), but still appears too low to stabilize the deaminase in vivo, if the data obtained in vitro (Table I) can be used as a guide. Furthermore, we have not yet been able to increase deaminase activity in normal liver appreciably in vivo by injecting thymine and cytosine deoxyribonucleosides or deoxyribonucleotides (1–7 mmoles/kg injected intraperitoneally 2–24 hours before assay), even though the thymidylate kinase activity of the liver can be stabilized under the same conditions (10). It seems probable, therefore, that the deaminase levels in the tissues depend on other factors in addition to the stabilizing action of deoxyribonucleotides, e.g., on the ability of the tissues to synthesize this enzyme.

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